Fazlul H. Sarkar *Editor*

Epigenetics and Cancer

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Editor
Fazlul H. Sarkar
Departments of Pathology
Barbara Ann Karmanos Cancer Institute
740 Hudson Webber Cancer Research
Wayne State University School of Medicine
Detroit, MI, USA

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Preface

I would like to thank the Springer publishing group for their faith and trust in me to organize the special topic on the emerging role of epigenetics in cancer development and progression in the book entitled *Epigenetics and Cancer*. This book illustrates the complexities of the regulation and deregulation of genes in the development and progression of human malignancies through epigenetics. It is well known that genetic aberrations, especially inherited through parents (somatic genetic alterations), contribute to the development of less than 10 % of all cancers, yet epigenetic alterations in genes are responsible for the development and progression of the vast majority of all cancers. Among many alterations in the expression of genes, epigenetic regulation of genes, especially through selective methylation and acetylation, appears to play an important role in the development and progression of human cancers. Understanding the role of epigenetics in the regulation of genes, especially through deregulated expression of microRNAs (miRNAs), will allow scientists to devise targeted therapeutic strategies for re-expression of the lost genes or down-regulating the genes that are over-expressed in order to eradicate cancer. It is hoped that targeting epigenetics will not only target cancer cells, but it will also target the tumor microenvironment (more like the entire tumor environment such as the entire host) for achieving better treatment outcomes for patients diagnosed with cancer toward the objective of complete eradication of cancer.

This book contains 15 chapters – which begins with the concept of systems and network biology for investigating the epigenetics of cancer, which has been well summarized by Muqbil et al. from Dr. Azmi's group – illustrating that an integrated approach of systems biology and network modeling would be important for investigating the role of miRNAs and their target genes in the biology of pancreatic cancer. This could indeed be applicable for all cancers, and such a strategy will allow the development of miRNA-targeting therapeutics as part of the personalized medicine for the treatment of human malignancies. This chapter is followed by the chapter on the role of miRNAs in cancer epigenetics by Dr. Fabbri's laboratory, and then a specific example on the epigenetic regulation of EZH2 and its targeted miRNAs has been documented in the chapter from Dr. Wong's laboratory. The next three chapters are focused on the epigenetic regulation of miRNAs. The first one is on the

epigenetics and miRNAs in renal cancer contributed by Majid et al., followed by the next chapter from Dr. Khare's laboratory, documenting epigenetic regulation of miRNAs in colon cancer. The third chapter is presented by Dr. Dong's laboratory, documenting the state of our knowledge on the epigenetic regulation of miRNAs in breast cancer development and progression.

It is becoming increasingly clear that cancer stem cells (CSCs) are important in the development and progression of cancer, and CSCs are important in therapeutic resistance, treatment failure and tumor recurrence. To highlight the importance of CSCs and epigenetics, Dr. Houchen's laboratory contributed a very timely chapter on the epigenetic variations of stem cell markers in cancer, including miRNAs. Subsequently, two chapters are focused on prostate cancer epigenetics: one is contributed by the laboratory of Dr. Bianco-Miotto on recent updates on epigenetic biomarkers for prostate cancer and the second one is by Dr. Rybicki describing the epigenetics and racial disparities in prostate cancer.

Emerging evidence suggests that epigenetic modifiers could be useful for cancer therapy as documented by the above chapters, and it is becoming increasingly clear that epigenetics plays an important role in the expression of genes including miRNAs, and thus finding novel strategies by which one could up-regulate or down-regulate genes and miRNAs through epigenetic-targeting agents would be welcome news for patients diagnosed with cancer. In order to provide state of our knowledge on epigenetic therapeutics, an example on the role of epigenetics and breast cancer is presented by Dr. Anant who summarized the state of our knowledge on the current drugs for targeting epigenetics that are in the drug pipeline. In the next chapter, Dr. Jazirehi's laboratory has summarized epigenetics in the context of immunotherapy and BRAF kinase inhibitor in the chapter entitled "Exploiting Epigenetic Modifiers to Circumvent Melanoma Dual Resistance to TCR-Engineered Immunotherapy- and $BRAF^{V600E}$-Kinase Inhibitor". This is followed by another therapeutic chapter on radiation therapy and epigenetics, which is a novel area of research as documented by Dr. Zielske in his chapter entitled "The Role of Epigenetics in Radiation Therapy and the DNA Damage Response".

There exists some novel agents that could target epigenetics in the therapeutic settings, but many such agents as presented above have already shown limitation because of unwanted adverse systemic toxicity. Therefore, further efforts are underway for testing the role of natural agents as possible non-toxic epigenetic-targeted therapeutics. This concept is presented by an exciting chapter from Dr. Gupta's laboratory, which documented that natural agents (cancer chemopreventive agents) could serve as epigenetic modifiers in the chapter entitled "Plant Polyphenols as Epigenetic Modulators of Glutathione *s*-transferase p1 Activity". Next, Dr. Li has provided a comprehensive view by describing the state of our knowledge on the epigenetic regulation of genes by natural agents (nutraceuticals) in the chapter entitled "Epigenetic Regulations of mRNAs and miRNAs by Nutraceuticals", which clearly suggests that selected nutraceutical agents could be useful as novel epigenetic-targeted therapeutic agents for the deregulation of specific genes, because nutraceuticals by definition are non-toxic to humans. Therefore, these agents could be administered safely and easily either alone or in combination

with conventional therapeutics to achieve better treatment outcomes for patients diagnosed with cancer.

It is now becoming increasingly clear that for cancer therapy to be a success, one must consider several aspects such as targeted agents for genes that are mutated, amplified or over-expressed in cancer cells, but targeting epithelial cancer cells only may not be the optimal therapeutic strategy. For that reason, drugs must be developed, which will also target cells that have undergone epithelial-to-mesenchymal transition (EMT phenotypic cells) as well as CSCs. Moreover, just targeting cancer cells, although they are heterogeneous, may still not be optimal to eradicate tumors, and for this one must take a holistic approach for developing drugs that could also target the tumor microenvironment and tumor dormancy that are regulated through epigenetics. Keeping abreast with this thought process, the concluding chapter contributed by the laboratory of Dr. Sheng provides such a concept in the chapter entitled "Towards Curative Cancer Therapy with Maspin: A Unique Window of Opportunity to Target Dormancy". This provides an example, but similar strategies could be developed for targeting the tumor dormancy and the tumor microenvironment by developing drugs that will specifically target epigenetics.

This book provides tip of the iceberg collection of articles on the state of our knowledge on epigenetics and cancer, which would likely be useful for bringing newer generations with broader perspectives in launching cutting-edge innovative molecular research that will certainly help in designing targeted clinical trials in order to realize the dream of tailored therapeutic approach for the prevention and/or treatment of human malignancies without causing any systemic toxicity. Moreover, the knowledge gained would allow novel utilization of agents, such as nutraceuticals, as adjunct to both conventional chemotherapy and radiation therapy in order to improve the overall quality of life and survival of patients diagnosed with cancer.

Lastly, I would like to thank all the authors for their cooperation, hard work and talented contributions to bring this book to the readers in a timely fashion, and I sincerely believe that the content of this book will be useful in educating young scientists so that they can carry the torch in innovative research for realizing the benefits of epigenetic targeting in the treatment of human malignancies. I would also like to thank the publisher and the entire publishing group for their dedication and professionalism. Finally, I would like to dedicate this book to my family for their understanding, unconditional support and sacrifice to enhance my profession as a scientist.

Wayne State University School of Medicine
Detroit, MI, USA

Fazlul H. Sarkar

Contents

Chapter 1
Systems and Network Biology to Investigate Epigenetic De-regulatory Mechanisms of MicroRNAs in Pancreatic Cancer

Irfana Muqbil, Fazlul H. Sarkar, Ramzi M. Mohammad, and Asfar S. Azmi

Abstract Pancreatic cancer (PC) is highly resistant to conventional therapeutics, and it is a complex disease which is partly characterized by genetic and epigenetic de-regulation of many signaling pathways. Among the numerous de-regulated mechanisms aberrant expression of small non-coding MicroRNAs (miRNAs) are very important. Intense research in this area has lead to the identification and characterization of critically de-regulated miRNAs which raises our hope that targeting them may lead to clinically beneficial outcome of patients diagnosed with PC. Emerging evidence suggests that miRNAs are under a highly coordinated epigenetic regulation, which could be exploited for the development of novel therapeutics. For the success of miRNA based therapeutic regimens, a holistic approach may be required that takes into account the emerging epigenetic regulatory mechanisms. In this chapter, the aberrant epigenetic grooming of miRNAs especially in PC and computational strategies especially systems and network biology to target them are discussed.

I. Muqbil
Department of Biochemistry, Faculty of Life Sciences,
AMU, Aligarh, India

F.H. Sarkar
Department of Pathology, Barbara Ann Karmanos Cancer Institute,
740 Hudson Webber Cancer Research Center, Wayne State University
School of Medicine, 4100 John R, Detroit, MI 48201, USA

R.M. Mohammad
Department of Oncology, Karmanos Cancer Institute, Wayne State University,
Detroit, MI 48201, USA

Hamad Medical Corporation, Doha, Qatar

A.S. Azmi, Ph.D. (✉)
Department of Pathology, Wayne State University School of Medicine,
Wayne State University, Detroit, MI 48201, USA
e-mail: azmia@karmanos.org

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1.1 Introduction

Pancreatic cancer (PC) is a deadly disease with estimated 168,800 annual deaths worldwide translating to ~20 deaths every hour (Global Cancer Statistics 2012) [1]. PC is diagnosed at a very late stage when it is refractory to surgery or standard chemotherapy [2]. The median survival is 6 months and overall therapeutic response rate is less than 5 % [3]. These dismal statistics indicate that newer diagnostic biomarkers and therapeutically druggable avenues need to be urgently identified. PC is among the most complex and heterogeneous of malignancies carrying multiple de-regulatory signaling mechanisms [4]. Among the various critical pathways found to be altered in PC, the microRNA (miRNA) system are being well recognized [5]. The miRNAs comprise a class of short noncoding RNAs that are 18–25 nucleotides in length and they are found in all animal and plant cells. In 1993, the first miRNAs were recognized in Caenorhabditis elegans by Lee et al. [6]. In 2001, various small regulatory RNAs were discovered in plants and mammals and designated 'microRNA' [7–9]. Currently, >1,200 human miRNAs are registered in the miRBase database [10]. The miRNAs have been extensively studied for their involvement in RNA interference (RNAi) machinery to regulate gene expression post-transcriptionally, and they are known to contribute to diverse physiological and pathophysiological functions, including the regulation of developmental timing and pattern formation, restriction of differentiation potential [10], cell signaling [11], cardiovascular diseases [12] and carcinogenesis [13]. The biogenesis and RNAi functions of miRNA (i.e. how miRNAs are generated and processed into a mature form, and how they regulate gene expression) have been intensively investigated and well-described [14]. Furthermore, developments in miRNA-related technologies, such as miRNA expression profiling and synthetic oligoRNA, have contributed to the identification of miRNAs that are known to be involved in a number of physiological and pathological phenotypes. A Pubmed search for microRNAs returns >19,900 hits similarly key words microRNA and cancer return >4,500 research articles. Most interestingly, an evaluation of research publications from 2000 to 2011 shows an exponential increase in research (summarized in Fig. 1.1) indicating that the field is advancing rapidly. These studies have led to much deeper understanding of microRNA biogenesis, their regulatory control on different genes and strategies to target them for anti-cancer therapeutic benefits. However, some questions remain largely unanswered, such as how miRNA expression is controlled and which genes are regulated by each miRNA in a specific disease condition.

The revolution of epigenetics has revitalized cancer research, shifting focus away from somatic mutation toward a more holistic perspective involving the dynamic states of chromatin. Disruption of chromatin organization can directly and indirectly precipitate in genomic instability and transformation. Not surprisingly,

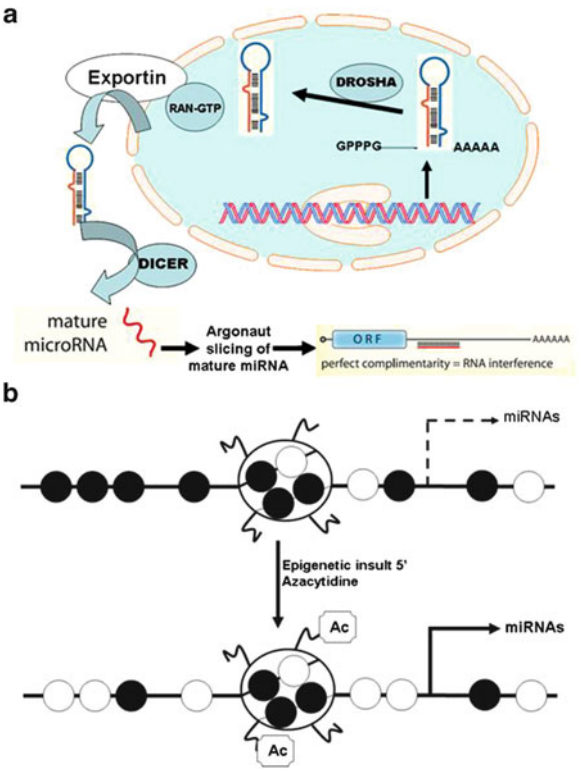

Fig. 1.1 miRNA biogenesis and epigenetic regulation: Generalization of a model involving epigenetic regulation of miRNAs in determining cell fate. A miRNA may be epigenetically silenced in the undifferentiated cell state by the combination of a methyl-DNA binding protein and associated chromatin-modifying repressor complex. In this state, the expression of the repressed miRNA is low whereas the expression of target mRNA transcript or protein coded for by the target transcript is high relative to the differentiated state. Upon an extrinsic or intrinsic signal cue for cellular differentiation, epigenetic repression of the target miRNA is released, and miRNA expression increases. The increase in miRNA expression then correlates with decreased stability or translation of target mRNAs. By epigenetically regulating one miRNA, the cell can thereby direct and fine-tune the expression of multiple miRNA-targeted mRNA transcripts during cell fate determination. The illustrated example provides a single direction in which this mechanism may potentially function, and it may be equally likely that the mechanism contributes to cell fate determination in the opposite manner as well. In this case, miRNA expression may become epigenetically silenced during differentiation. Subsequently, target mRNA translation would increase and proteins important for the differentiating cells would be expressed at higher levels than the undifferentiated cells

studies have shown that a greater majority of miRNAs are regulated epigenetically [15]. There are some studies that have focused upon the relationship between miRNA and its consequence on therapy resistance especially in complex diseases such as PC. We will first illustrate the current knowledge regarding the epigenetics–miRNA regulatory networks and its impact on inducing drug resistance in PC.

1.2 MicroRNA Biogenesis and Their Epigenetic Regulatory Mechanisms

Over the years, our understanding of microRNAs biogenesis has increased and this is in part due to increased understanding of the transcription, replication and translations process. The miRNA biogenesis originates primarily in the nucleus where the RNA polymerase II initially transcribes miRNAs into long segments of coding or noncoding RNA, known as pri-miRNAs that carry a polyadenylated cap. Following this, RNase type III, Drosha and the dsRNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8) (also called Pasha) (collectively called as microprocessor complex) capture short sets of the pri-miRNAs measuring approximately 70–100 nucleotides in length and containing a stem-loop becomes pre-miRNAs [19]. Pre-miRNAs form a complex with exportin-5 (XPO-5) and RAN-GTP, and undergo nuclear export to cytoplasm. Following this a Dicer or type III dsRNAse further processes the pre-miRNAs to a double-stranded miRNA duplex (ds-miRNA). This ds-miRNA duplex is incorporated into a RNA-induced silencer complex (RISC)-loading complex (RLC) in an ATP-dependent manner [20]. One strand (the passenger strand) of the miRNA is removed from the RLC, whereas the other guide strand remains in the complex to form a mature RNA-induced silencer complex (RISC) and serves as a template for capturing target miRNAs. The mature RISC represses gene expression post-transcriptionally and the core (catalytic) component or RNase III domain of a Argonaute protein cleaves the RISC to generate highly complementary target miRNAs [21]. For partially complementary targets, the RISC complex deadenylates target miRNAs and this, in turn, reduces the stability of target microRNAs [22]. Additionally, the RISC complex has also been shown to repress the translation of target genes under most conditions (Biogenesis summarized in Fig. 1.1a). However, the mechanisms underlying miRNA turnover in human cells remains unclear. Instead of re-reviewing the existing knowledge on miRNA processing and their targets, the focus of this article is to highlight the disease specific role of miRNA epigenetics which is described below.

Even though the biogenesis of miRNA has been intensively studied and is well-described, the regulation of miRNA expression remains largely unclear. In early studies, promoter regions had been determined for only a small subset of miRNAs while in-silico predictive studies have provided blueprints of the promoter regions of miRNAs [16]. However most of these predicted miRNA promoters have yet to be confirmed in wet-laboratory experiments. The miRNAs are classified as either ‘intragenic’ or ‘intergenic’, according to whether the miRNA is localized in a genomic region transcribed by a gene, or not. The means by which miRNA expression is regulated appears somewhat complicated. Earlier, it was established that epigenetics controls a number of genes in cancer and other diseases while recent evidence shows that epigenetics and miRNAs control each other forming a regulatory circuit that maintains normal physiological functions. One can envision that a disruption of this regulatory circuit can manifest into various diseases, such as cardiovascular diseases and cancer.

The earliest study on epigenetic control of miRNA were published in seminal paper by Saito and colleagues where it was established that the expression of miR-127 is regulated epigenetically [17]. In this study, using a bladder cancer cell model, it was shown that targeted demethylation resulted in the activation of certain microRNAs (miR-127). Specifically, the DNA methylation level and histone modification status at identified promoter regions of miR-127 was correlated significantly with mature miR-127 expression. These findings paved the way for numerous subsequent studies documenting epigenetic modulation of miRNAs in different cancer models.

1.3 Epigenetic Modulation of miRNAs in PC

With respect to PC, a number of miRNAs have been correlated to drug resistance and poor overall survival. Our laboratory was among the first to demonstrate the expression profile of microRNAs (miRNAs) in the plasma of patients diagnosed with PC (n = 50) compared with healthy volunteers (n = 10) [18]. In this study, 37 different miRNAs were found to be down-regulated and 54 were up-regulated in the plasma from patients with PC. The expression of miR-21 was significantly higher, and the expression of let-7 family (especially let-7d) and miR-146a was significantly lower in PC. Most interestingly, the expression of miR-21 was correlated with poor overall survival, and the expression of let-7 was inversely correlated with survival in this pilot study with mixed patient population. Additionally, we observed miR-21 family was markedly over-expressed in chemo-resistant PC cell line models, which was consistent with the plasma data from PC patients. This was a proof of principle study suggesting that identifying and validating the expression of miRNAs in newly diagnosed patients could possibly serve as potential biomarker for tumor aggressiveness, and such miRNAs could be useful for the screening of high-risk patients, and may also serve as targets for future drug development. We have also verified the de-regulated miRNA signatures in RNA samples derived from fine needle aspirate of PC patients [19]. These studies have verified the role of deregulated miRNAs and their association with deregulated signaling in PC. The next question is how miRNA expression is deregulated, which is discussed in the following paragraphs.

The first study on miRNA epigenetic regulation in PC came from Maitra's group where they have used two human pancreatic cancer cell lines – MiaPACA-2 and PANC-1 to verify the effect of demethylating agents, 5-aza-2′-deoxycytidine (5-Aza-dC) or the histone deacetylase inhibitor, trichostatin A, as well as the combination of the two [20]. Control and treated cell lines were assessed using a custom microarray platform. Fourteen miRNAs were found to be up-regulated by two-fold or greater in each of the cell lines following exposure to both chromatin-modifying agents, including five miRNAs that were found to be common (miR-107, miR-103, miR-29a, miR-29b, and miR-320) to both MiaPACA-2 and PANC-1 cell lines. The differential over expression of miR-107 in the treated cancer cell lines was

confirmed by Northern blot assays. Methylation-specific PCR assays for assessment of CpG island methylation status in the 5′ promoter region of the miR-107 primary transcript demonstrated complete loss of methylation upon exposure to 5-Aza-dC. Interestingly, forced expression of miR-107 in MiaPACA-2 and PANC-1 cells reduced *in vitro* growth, and this was associated with repression of the putative miR-107 target, cyclin-dependent kinase 6. These studies provided functional basis for the epigenetic inactivation of this miRNA in pancreatic cancer.

In another epigenetic study on miRNAs in PC, Mees and colleagues used a total of 16 human PDAC cell lines in murine orthotopic PDAC models [21]. Using a standardized dissemination score, local invasion and metastatic spread were assessed. The authors detected CD40 as a relevant target gene for differentially expressed miRNAs observed in highly invasive and metastatic PDAC only. A significant overexpression ($P < 0.05$) of CD40-related miRNAs such as miR-224 and miR-486 was detected in highly invasive and metastatic PDAC models, whereas CD40 mRNA expression was not significantly altered. Intriguingly, CD40 protein expression at cell surfaces of these highly invasive and metastatic PDAC was significantly reduced ($P < 0.01$). Most importantly, epigenetic alterations with up-regulated CD40-targeting miR-224 and miR-486 were found to be related to reduced-CD40 protein expression at cell surfaces in highly invasive and metastatic PDAC. From these studies, the authors have concluded that miRNA-regulated CD40 expression seems to play an important role in progression of PDAC. These data also suggested a diagnostic and therapeutic potential for CD40 and its targeting miRNAs in PDAC.

Recently Kitamoto and colleagues in their study presented epigenetic regulatory effects of miRNAs on Mucins (specifically, MUC17) that is recognized to be aberrantly expressed in PC [22]. Using gene expression analysis, epigenetic regulation investigations such as promoter methylation, histone modification and miRNA expression, the authors showed that near the transcriptional start site, the DNA methylation level of MUC17-negative cancer cell lines (e.g. PANC1) was high, whereas that of MUC17-positive cells (e.g. AsPC-1) was low. Histone H3-K9 (H3-K9) modification status was also closely related to MUC17 expression. Their results indicated that DNA methylation and histone H3-K9 modification in the 5′ flanking region could play a critical role in MUC17 expression. These cell line observations were correlated with hypomethylation status as observed in patients with PDAC. From these studies, it was concluded that the hypomethylation status in the MUC17 promoter could be a novel epigenetic marker for the diagnosis of PDAC. Most importantly, the result of miRNA microarray analysis showed that five potential miRNA candidates existed, suggesting that the MUC17 might be post-transcriptionally regulated by miRNA targeting to the 3′-untranslated region of its mRNA.

Apart from the above studies, miR-34 family has been well investigated for epigenetic regulations in PC. In this direction Vogt and colleagues found frequent concomitant inactivation of miR-34a and miR-34b/c by CpG methylation in PC and other solid tumors [23]. The authors proposed that miR-34a down-regulation and CpG methylation can serve as diagnostic marker for this disease. Supporting this study, Nalls and coworkers investigated the functional significance of miR-34a in

PC progression through its epigenetic restoration with chromatin modulators, demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC) and HDAC inhibitor Vorinostat (SAHA) [24]. The authors observed re-expression of miR-34a in human PC stem cells (CSCs) and in human PC cell lines upon treatment with 5-Aza-dC and SAHA which was strongly associated with inhibition of cell proliferation, cell cycle progression, self-renewal, epithelial to mesenchymal transition (EMT) and invasion. In PC CSCs, modulation of miR-34a induced apoptosis by activating caspase-3/7. Most interestingly, treatment of pancreatic CSCs with the chromatin-modulating agents resulted in the inhibition of Bcl-2, CDK6 and SIRT1, which are considered the putative targets of miR-34a. The miR-34a up-regulation by these agents also induced acetylated p53, p21(WAF1), p27(KIP1) and PUMA in pancreatic CSCs. Inhibition of miR-34a by antagomiR abrogated the effects of 5-Aza-dC and SAHA, suggesting that 5-Aza-dC and SAHA regulates stem cell characteristics through miR-34a.

In CSCs, SAHA inhibited Notch pathway, suggesting its suppression may contribute to the inhibition of the self-renewal capacity and induction of apoptosis. Interestingly, treatment of pancreatic CSCs with SAHA resulted in the inhibition of EMT with the transcriptional up-regulation of E-Cadherin and down-regulation of N-Cadherin. Expression of EMT inducers (Zeb-1, Snail and Slug) was inhibited in CSCs upon treatment with SAHA. The 5-Aza-dC and SAHA also retarded the *in vitro* migration and invasion of CSCs. From these comprehensive studies, the authors have demonstrated the role of miR-34a as a critical regulator of pancreatic cancer progression by regulating CSC characteristics. The restoration of its expression by 5-Aza-dC and SAHA in CSCs was suggested to not only provide mechanistic insight and therapeutic targets for PC but also identify promising methodologies to boost patient response to existing chemotherapies or as a stand-alone cancer drug by eliminating the CSC characteristics. Collectively, these studies have confirmed the critical role of epigenetics in the regulations of miRNAs in PC. In the following passages the emerging computational technologies that are helping in better understanding the complexity of epigenetic mechanism of miRNAs in cancer in general including PC is discussed.

1.4 Why Computational Approaches Are Needed to Study miRNA Epigenetics?

Till date most of the studies have employed differential expression of miRNAs to identify candidates for subsequent epigenetic evaluation. This approach introduces bias that limits the number of epigenetically regulated miRNAs that can be identified. For example, tissue-specific miRNAs that are influenced by DNA methylation may be equally methylated in normal tissues and tumors, resulting in a lack of differential expression between normal and cancerous specimens. Furthermore, miRNAs with low expression levels cannot be reliably identified because the differences in the expression between normal and cancer or between baseline and demethylated

conditions will likely fall below conventional cutoffs limit that is ideally set between 2 and 1.5 fold. Finally, residual methylation persists in both pharmacologically and genetically demethylated cells [25]. Such methylation may be critical in maintaining cell viability, potentially through persistent repression of key miRNAs. This would result in such miRNAs not being identified through the expression-based strategies.

As elegantly summarized by Griffith Jones and colleagues, there are three critical steps in the discovery of miRNA-mediated gene regulatory signaling namely (a) discover co-expressed genes; (b) discover conserved cis-regulatory signature in UTRs of co-expressed genes as evidence of miRNA-mediated control; and finally (c) identify the miRNA seed sequence from miRBase that is complementary to the *cis*-regulatory signature [26]. Baliga and co-workers have developed a very user friendly analysis system, miRvestigator that allow users the flexibility to use any method to select a gene set that are likely to be co-regulated by a common factor [27]. The miRvestigator uses a *de novo* motif discovery algorithm that models miRNA binding in a probabilistic manner. Unlike earlier algorithm models which worked on a sorted gene list, miRvestigator expects a specifically selected subset of co-expressed genes identified using classification methods such as hierarchical clustering, biclustering and others. The miRvestigator scans the 3′-UTR sequences of query genes for an overrepresented sequence motif using the Weeder software package.

The complexity of the action of miRNAs calls for comprehensive, integrative systems level approaches to examine the effect of miRNAs. To this end, genomic and genome-related approaches in the study of miRNA have been appreciated recently. Integration of genome-related approaches with physiological and clinical approaches has been shown to be valuable for further elucidating the role of miRNAs in systems and personalized medicine. Notably, advanced genomics, proteomic, epigenomic techniques have begun to be utilized in the analysis of widespread effects of miRNAs [28]. Other approaches that have been used include large-scale sequencing of miRNA and potential miRNA targets, mRNA expression profiling, and bio-informatics modeling [29]. Sequencing of cleaved fragments of mRNAs has been used to identify miRNA targets, the applicability of which would depend on the extent to which miRNAs induce mRNA cleavage in a given species.

To overcome the discovery bias introduced by expression-based identification strategy, hollistic analysis is needed that directly utilizes global DNA methylation patterns to identify miRNAs regulated by DNA methylation of cancer cells. This requires computational tools that can not only identify miRNAs related to disease but also meaningfully unwind the complex interactions they regulate that result in a disease phenotype (Fig. 1.2 showing the complexity of miRNA network exemplified by let7 miRNA). Initial computational studies were restricted to mapping genome-wide DNA methylation in cancer cell lines using methyl CpG binding domain (MBD)-isolated Genome Sequencing (MiGS) [30]. Additionally miRNAs with proximal DNA methylation as candidates have also been identified that were cross-referenced the list of candidates with miRNA expression data as supporting evidence. Building such mapping studies, researchers have been able to perform

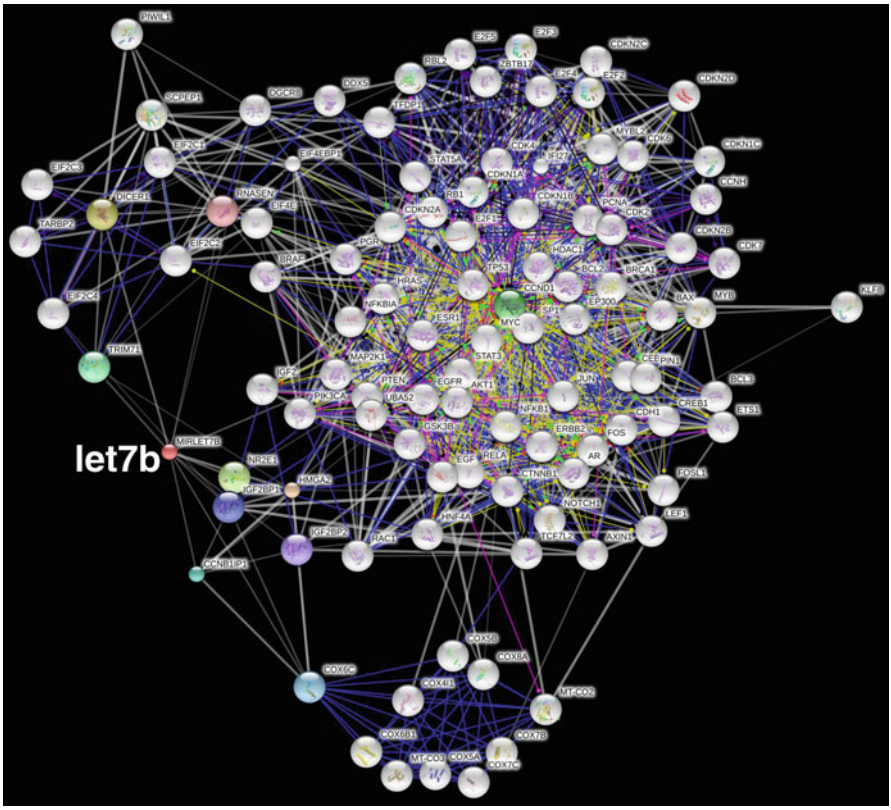

Fig. 1.2 Complex miRNA interaction network requires systems and network level understanding. A single miRNA can regulate and/or influence 100s of proteins. Figure showing interaction network of let7 miRNA (commonly found aberrantly expressed in different cancers). Nodes are either colored if they are directly linked to the input or white (nodes of a higher iteration/depth). Edges, i.e. predicted functional links, consist of up to eight lines: one color for each type of evidence. Network was developed using String 9.0

functional analysis of methylation regulated miRNAs in cancer. Using such approaches, Hongli Yan and colleagues have successfully identified both known and novel DNA methylation-regulated miRNAs [31]. They found 64 miRNAs to be robustly methylated in HCT116 cells; 18 of them were located in imprinting regions or already reported to be regulated by DNA methylation. For the remaining 46 miRNAs, expression levels of 18 were consistent with their DNA methylation status. Being consistent with their observations, another study showed that interacting proteins in the human PPI network tend to share restricted miRNA target-site types rather than random pairs [32]. Interestingly, a computational method named mirBridge that assesses enrichment of functional sites for a given miRNA in the annotated gene set showed that many epigenetically regulated miRNAs coordinately regulate multiple components of signaling pathways and protein complexes related to cancer [33].

Ju-Ichi Satoh and colleagues have identified a coordinated regulation of gene expression by transcription factors and miRNAs at transcriptional and epigenetic levels in cancer-associated miRNA targetome networks [34]. Importantly, a recent study showed that the genes with more transcription factor-binding sites have a higher probability of being targeted by epigenetically modified miRNAs regulations and have more miRNA-binding sites [35]. These observations support the general view that the human miRNAome and miRNA epigenome play specialized role in the regulation of oncogenesis. Therefore, the miRNA-based therapy designed to simultaneously target multiple cancer-associated networks and pathways might serve as the most effective approach in suppressing the oncogenic potential in a wide range of cancers. This can only be achieved if computational sciences are taken into consideration for the design of therapeutics strategies involving these multi-targeted small molecules.

1.5 Conclusions

PC is a deadly disease that is considered by far incurable among all other human malignancies. The disease is in a dire need of modern therapeutics as well as technologies to better understand the basic tenets of its origin. The miRNAs have been touted as the future of personalized therapy against cancer including PC and a number of miRNA targeted strategies are advancing towards clinical assessment for different cancers. However, a number of challenges remain especially with the discovery of epigenetic regulation of miRNAs that adds to a second tier of regulatory control on these small molecules. This is in addition to the by far incomprehensive level of complexity involved in the number of targets each miRNA regulates. Such large scale interaction networks can not be feasibly evaluated using reductionist molecular biology approach. Therefore, computational approaches would allow interrogating a variety of biological knowledge that can be extracted from the complexity of miRNA interactions. Integrated analysis of expression patterns comes in handy when the molecules under study have complex biological functions, such as those of miRNAs. Since the target mRNAs are regulated in a ‘one-to-many’ and a ‘many-to-one’ manner and the degree of regulation vary case-by-case or becomes context dependent. When accumulated miRNA–mRNA interactions identify biological functions, it will be necessary to look at those interactions comprehensively and recognize them as part of a gene regulatory network. Therefore, we suggest that further weight should be given to high-throughput analyses combined with computational approaches, as an effective methodology to achieve a systems-level understanding of complex biological functions of epigenetically groomed miRNAs. This will aid in the design of newer strategies that could selectively modulate the miRNAs for therapeutic benefit in order to realizing the dream of better and improved treatment outcome of patients diagnosed with deadly diseases such as PC.

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Chapter 2
Role of MicroRNAs in Cancer Epigenetics

Kishore B. Challagundla, Petra Wise, and Muller Fabbri

Abstract MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) with gene expression regulatory functions. Increasing evidence shows that, despite not translated, miRNAs undergo the same regulatory mechanisms of any other protein coding gene (PCG). In particular, they undergo epigenetic regulation. Intriguingly, cancer cells are able to epigenetically regulate the expression of selected miRNAs, therefore granting an overall shift of the transcriptome towards an oncogenic phenotype. In parallel, miRNAs also directly target the expression of key effectors of the epigenetic machinery, therefore indirectly modulating the expression of epigenetically controlled PCGs. This intertwined relationship between the miRNome and the epigenome is further complicated by the existence of other categories of ncRNAs, also modulated by miRNAs and their epigenetic interactions. Overall, the complex layers of reciprocal regulation between ncRNAs and epigenetics are discussed in this chapter and represent a fundamental aspect of the biology of cancer cells.

Keywords Epigenetics • Cancer • MicroRNAs • Non-coding RNAs • Methylation • Chromatin • Histone • Oncogene • Tumor suppressor gene • Promoter • DNA methyltransferases • Histone deacetylases • Polycomb • Post-transcriptional regulation • Gene expression

K.B. Challagundla • P. Wise • M. Fabbri, MD, Ph.D. (✉)
Children's Center for Cancer and Blood Diseases, Norris Comprehensive Cancer Center,
Children's Hospital Los Angeles, University of Southern California,
4650 Sunset Blvd, MS #57, Los Angeles, CA 90027, USA
e-mail: mfabbri@chla.usc.edu

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2.1 Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) which regulate gene expression at a post-transcriptional level [1]. MiRNA aberrant expression is involved in the genesis of several human diseases, including cancer [2]. Interestingly, it has been shown that the genes encoding for miRNAs undergo the same epigenetic regulation of any other protein coding gene (PCG), namely promoter methylation, histone acetylation and chromatin changes [3]. In addition, miRNAs can modulate the expression of key effectors of the epigenetic machinery, such as DNA methyltransferases (DNMTs), Histone deacetylases (HDACs), Polycomb genes, etc.… [4]. Recently, it has been shown that other ncRNAs, namely the transcribed ultraconserved regions (T-UCRs), are also dys-regulated in cancer, and their expression is controlled by miRNAs [5]. This discovery has provided the first evidence of a reciprocal epigenetic control between two different categories of ncRNAs. We define this interaction as direct epigenetic control of ncRNAs. Moreover, increasing evidence is showing that miRNAs are involved in feedback and feedforward regulatory loop, responsible for key steps in human carcinogenesis and drug resistance development. In some cases, it even has been shown that specific miRNAs can regulate the expression of other miRNAs through a common molecular pathway involving transcription factors [6]. We define this interaction as indirect epigenetic control of ncRNAs. This chapter will focus on these interactions by showing at first which miRNAs undergo an epigenetic control in some of the most common human malignancies, followed by a description of which miRNAs directly target key effectors of the epigenetic machinery. Finally, we will describe the direct and indirect mechanism through which miRNAs modulate other ncRNA expression.

2.2 Epigenetic Regulation of MicroRNAs in Human Cancer

2.2.1 Breast Cancer

Epigenetic regulation is responsible for aberrant miRNA expression in several malignancies. One of the first studies in this field was conducted in a breast cancer cell line by Scott et al. [7] who were able to demonstrate that 27 miRNA expression levels are rapidly modified by treatment with the HDAC inhibitor LAQ824, indicating that indeed epigenetic factors are involved in miRNA regulation [7]. In breast cancer cell lines treated with 5-aza-2′-deoxycytidine (5-AZA), a DNA demethylating agent, a reactivation of miR-9-1 occurred, without changes in the levels of the other aberrantly methylated miRNAs [8], suggesting that different epigenetic processes can control epigenetically regulated miRNAs in different types of cancer. Tavazoie et al. showed that miR-335, miR-206 and miR-126 act as metastasis suppressors and their expression levels are significantly reduced in primary breast neoplasms of patients who developed metastases [9]. In the case of miR-335 this reduction of expression was

partially due to a locus deletion in combination with hypermethylation of the miR-335 promoter region. The "maintenance" DNA methyltransferase 1 (DNMT1) was found to be aberrantly upregulated in breast cancer and was responsible for hypermethylation of miR-148a and miR-152 promoter regions. DNMT1 expression, one of the targets of miR-148a/152, was inversely correlated with the expression levels of miR-148a/152 in breast cancer tissues, suggesting a negative feedback regulatory loop [10]. Interestingly, IGF-IR and IRS1, often overexpressed in breast cancer, were also targets of miR-148a/152. Overexpression of miR-148a or miR-152 significantly inhibited cell proliferation, colony formation, and tumor angiogenesis *via* targeting IGF-IR and IRS1 and suppressing their downstream AKT and MAPK/ERK signaling pathways [10]. Chang and Sharan reported that BRCA1 recruits the HDAC2 complex to the miR-155 promoter, which is consequently epigenetically silenced through the deacetylation of H2A and H3 histones [11]. The study also showed the up-regulation of miR-155 in BRCA1 deficient or BRCA1 mutant human tumors. The knockdown of miR-155 in a BRCA1 mutant tumor cell line attenuates *in vivo* tumor growth. However, a knockdown of BRCA1 results in a twofold to threefold increase in miR-155 levels *in vitro*. In contrast, a 50 to 150-fold increase in miR-155 in human breast cancer cell lines or tumor samples was observed suggesting that this increase may not be caused only by BRCA1 loss; other transcription factors may also activate the miR-155 promoter after it is epigenetically activated due to the loss of BRCA1 [11].

2.2.2 Colorectal Cancer

Lujambio et al. created a double knockout (DKO) for DNMT1 and DNMT3b in the colorectal cancer cell line HCT-116 and compared the miRNA expression profile of DKO and wild-type cells [12]. About 6 % of the 320 analyzed miRNAs were upregulated in the DKO cells. Among the dysregulated miRNAs, only miR-124a was embedded in a CpG island that is densely methylated in the cancer cell line, but not in normal tissue. This miRNA directly targets CDK6, and its restoration reduces the levels of CDK6 and has an impact on the phosphorylation status of the CDK6 downstream effector Rb protein [12]. The miR-34b/c cluster as well is epigenetically regulated in colorectal cancer; Toyota et al. [13] demonstrated a promoter hypermethylation in 90 % of primary colorectal cancer tumors versus normal colon mucosa. The relationship between miRNA and cognate host gene epigenetic regulation was addressed by Grady et al. [14] by studying miR-342, located in an intron of the EVL (Ena/Vasp-like) gene. EVL promoter hypermethylation occurs in 86 % of colorectal cancers and is already present in 67 % of adenomas, suggesting that it is an early event in colon carcinogenesis. A combined treatment of 5-AZA with the HDAC inhibitor trichostatin A restores the synchronized expression of EVL and miR-342 [14]. In samples from patients with colorectal cancer, 5 miRNAs were identified that were down-regulated and located around/on a CpG island. Treatment with 5-AZA and the HDAC inhibitor 4-phenylbutyric acid restored expression of 3 of the 5 microRNAs (namely miR-9, miR-129 and miR-137) in 3 CRC cell lines.

Expression of miR-9 was inversely correlated with methylation of their promoter regions [15]. Further, methylation of the miR-9-1, miR-129-2 and miR-137 CpG islands were observed in CRC cell lines and in primary CRC tumors, but not in normal colonic mucosa. The methylation of miR-9-1 was associated with the presence of lymph node metastasis [15]. After screening 64 potential epigenetically regulated miRNAs in colon cancer cells, Yan et al. identified miR-941, miR-1237 and miR-1247 as upregulated after treatment of the cells with 5-AZA and transcriptionally independent from their respective putative host genes [16]. Functional studies of miR-941 and miR-1247 revealed that both miRNAs suppress cell growth and migration in CRC cells. Ectopic expression of miR-1247 significantly reduced cancer cell proliferation and migration in colon cancer cells, suggesting that miR-1247 may function as a tumor suppressor gene [16].

DNA methylation also regulates the expression of the miR-1-1 and miR-133a-2 cluster in CRC cell lines. After examining the expression of miR-1 and miR-133a in 64 paired tissue samples (CRC tumor and adjacent normal mucosa), Chen et al. found that the miR-1-133a cluster displayed significantly lower expression in CRC tissue compared to adjacent normal mucosa [17]. The results indicated frequent hypermethylation of the CpG islands upstream of miR-1-133a; liver metastatic tissues exhibited significantly lower miR-1 and miR-133a expression compared to adjacent normal mucosa. The expression of the miR-1-133a cluster is inversely correlated with TAGLN2 in the tested tumor specimens; therefore, epigenetic repression of the miR-1-133a cluster may play a critical role in colorectal cancer metastasis by silencing TAGLN2 [17]. Vinci et al. evaluated the expression of the miR-9-1 and miR-34b/c in CRC paired tissue samples from 160 patients and reported in all cases a significantly reduced expression miR-34c and miR-9-1 [18]. Subsequently, the analysis of the level of methylation in CRC and normal tissues revealed significant hypermethylation in tumor tissues for both miR-34b/c and miR-9-1 [18].

2.2.3 Lung Cancer

In HCT-116 cells deficient in DNMT1 and DNMT3B, Brueckner et al. demonstrated increased expression of let-7a-3, a miRNA normally silenced by promoter hypermethylation in the wildtype cell line [19]. In lung adenocarcinoma primary tumors, let-7a-3 promoter was found hypomethylated with respect to the normal counterpart [19], whereas hypermethylation of let-7a-3 promoter was described in epithelial ovarian cancer, paralleled the low expression of insulin-like growth factor-II expression, and was associated with a good prognosis [20]. Therefore, DNA methylation could act as a protective mechanism by silencing miRNAs that have oncogenic function.

The above-mentioned studies demonstrate that epigenetic factors can control human carcinogenesis, not only by directly affecting the expression of oncogenes (OGs) and tumor suppressor genes (TSGs), but also by affecting the expression of miRNAs involved in oncogenic pathways. MiRNA epigenetic control might be tissue-specific because no variation in miRNA expression was observed in lung cancer cells treated with either demethylating agents or HDAC inhibitors or their combination [21].

Besides via DNA methylation, epigenetic silencing in mammalian cells can also be mediated by histone modifications. For instance, increased levels of H3K27 trimethylation and H3K9 dimethylation as well as H3K9 acetylation in the promoter region of miR-212 in lung cancer cells compared to normal cells was observed [22], leading to a reduced expression of miR-212 in lung cancer compared to the normal lung tissue counterpart [23].

2.2.4 Hepatocellular Carcinoma (HCC)

In HCC miR-1 is frequently silenced by promoter hypermethylation. However, in DNMT1-null HCT-116 cells (but not in DNMT3B-null cells), hypomethylation and re-expression of miR-1-1 were observed [24], revealing a key role for the maintenance DNMT in the regulation of this miRNA. Aberrations in histone acetylation have been observed in HCC. In their study, Yuan et al. [25] determined that miR-200a and the level of histone H3 acetylation at its promoter region were reduced in human HCC tissues in comparison with adjacent noncancerous hepatic tissues. Furthermore, histone deacetylase 4 (HDAC4) inhibited the expression of miR-200a and its promoter activity and reduced the histone H3 acetylation level at the mir-200a promoter region through a Sp1-dependent pathway. Interestingly, the miR-200a directly targeted the 3′-untranslated region of the HDAC4 messenger RNA and repressed expression of HDAC4. This means that miR-200a ultimately induced its own transcription and increased the histone H3 acetylation level at its own promoter. After screening 78 HCC patient tissue samples, He et al. found miR-191 to be highly expressed in tumor tissues and the adjacent noncancerous tissues compared to normal liver [26]. This elevated expression was associated with poor prognosis: mir-191 overexpression led to a mesenchymal-like transition, and increased cell invasion. The mir-191 locus is located in the gene *DALRD3*, with which mir-191 is co-expressed. The *DALRD3* promoter region contains a CpG rich region that is hypomethylated in HCC. Treatment of normal liver cells with 5-AZA showed an increase in miR-191 expression, which suggests that mir-191 is involved in HCC progression [26]. Also, miR-224 is commonly upregulated in HCC, and regulates apoptosis and cell proliferation. Wang et al. [27] examined the expression of miR-224, neighboring miR-452 and genes on chromosome Xq28 in paired tissues from patients with HCC, finding that miR-224 is coordinately upregulated with its neighboring miRNAs and genes. The introduction of histone deacetylase (HDAC) inhibitors in non-transformed human liver cells resulted in a corresponding increase in histone H3 acetylation in this region. MiR-224 locus in Xq28 resulted reciprocally regulated by HDAC1, HDAC3, and histone acetylase protein, E1A binding protein p300 (EP300). Notably, in HCC tumors significantly overexpressing miR-224, EP300 is also overexpressed and displays increased binding to the Xq28 locus. Through inhibition of EP300 the high miR-224 expression in transformed HCC cells can be attenuated [27]. Liu et al. reported that a large Chromosome 19 miRNA cluster (C19MC) is upregulated in HCC cells after combined treatment with 5-AZA and trichostatin A [28]. Specifically, miR-517a and miR-517c were strikingly different from the

remaining 41 miRNAs in C19MC. Ectopic expression of miR-517a and miR-517c inhibited cell proliferation by blocking G2/M transition, whereas downregulation of miR-517a and miR-517c facilitated cell growth. The group showed that Pyk2 is a target of miR-517a/517c and both miRNAs are downregulated in HCC samples. These data collectively suggest that downregulation of both miR-517a and miR-517c contributes to HCC development by regulating Pyk2 [28].

2.2.5 Melanoma

Mazar et al. studied which miRNAs were upregulated upon treatment of a melanoma cell line with demethylating agents [29]. Among the 15 miRNAs silenced by promoter hyper-methylation, they showed that miR-375 and miR-34b are also involved in melanoma progression [29]. To investigate the epigenetic regulation of miRNAs in melanoma, Liu et al. [30] found that miR-182, a miRNA with oncogenic properties, was significantly upregulated in human melanoma cells after epigenetic modulation with 5-AZA and trichostatin A. Genome sequence analysis revealed the presence of a prominent CpG island 8–10 kb upstream of miR-182, whereas methylation analysis showed that this genomic region was exclusively methylated in melanoma cells but not in human melanocytes, skin, or peripheral blood mononuclear cells. This increased expression of the oncogenic miR-182 could be a concern for melanoma patients after epigenetic therapy [30].

The genomic region on chromosome 9p21 where miR-31 is located, is frequently deleted in solid cancers including melanoma. Asangani et al. [31] found that downregulation of miR-31 was a common event in melanoma primary tumors and cell lines and was associated with genomic loss in a subset of samples as well as with epigenetic silencing by DNA methylation and EZH2-mediated histone methylation. Ectopic overexpression of miR-31 in various melanoma cell lines inhibited cell migration and invasiveness. MiR-31 target genes included oncogenic kinases such as SRC, MET, NIK (MAP3K14) and the melanoma specific oncogene RAB27a. Furthermore, miR-31 overexpression resulted in downregulation of EZH2 and a repression of its target gene rap1GAP. The increased expression of EZH2 was associated with melanoma progression and poorer overall survival. Taken together, these data support a tumor suppressor role for miR-31 in melanoma and might identify potential novel therapeutic targets [31].

2.2.6 Leukemias

Prosper's group analyzed 353 acute lymphoblastic leukemia (ALL) patients and identified a signature of 13 miRNAs embedded in CpG islands, with high heterochromatic markers (namely, high levels of K9H3me2 and/or low levels of K4H3me3) [32, 33]. Treatment with 5-AZA induced upregulation of at least one miRNA of the signature

in 65 % of ALL patients [33]. Among these, miR-124a was methylated in 59 % of ALL patients, and its promoter hypermethylation was associated with a higher relapse and mortality rate versus non-hypermethylated cases [32]. Additionally, the impact of miR-124a in the CDK6-Rb pathway was demonstrated in ALL by showing that miR-124a directly silences CDK6 [32]. Rodriguez-Otero et al. analyzed the methylation status of the members of the miR-9 family, miR-9-1, miR-9-2 and miR-9-3, in a uniformly treated cohort of 200 newly diagnosed ALLs [34]. MiR-9 was methylated in 54 % of the patients and was associated with downregulation of miR-9 expression. Hypermethylation of miR-9 was an independent prognostic factor for disease-free survival, overall survival and event-free survival in a multivariate analysis. Epigenetic downregulation of miR-9 induced upregulation of its targets, FGFR1 and CDK6, while treatment of ALL cells with FGFR1 and CDK6 inhibitors induced a decrease in cell proliferation and increased apoptosis of ALL cells [34]. Transcription factors are able to recruit epigenetic effectors at miRNA promoter regions and contribute to the regulation of their expression as shown by Fazi et al. [35]. The AML1/ETO fusion oncoprotein is the aberrant product of t(8;21) translocation in acute myeloid leukemia (AML) and can bind to the pre-miR-223 region. The oncoprotein recruits epigenetic effectors (i.e., DNMTs, HDAC1, and MeCP2), leading to aberrant hypermethylation of the CpG in close proximity to the AML1/ETO binding site and H3-H4 deacetylation of the same chromatin region [35]. Finally, Chim et al. studied miR-34a, miR-124-1 and mir-203 in a panel of hematological malignancies [36–38] including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL) and non-Hodgkin's lymphoma (NHL). All three of the investigated miRNAs were found to be epigenetically silenced in a tumor specific manner: miR-34a methylation was detected in a percentage of CLL, MM and NHL samples at diagnosis but not at all in ALL, AML and CML. Amongst lymphoid malignancies, was miR-34a preferentially methylated in NHL, in particular in natural killer (NK)/T-cell lymphoma. Methylation of miR-124-1 as well as miR-203 could not be detected in CML but in ALL, AML, CLL and NHL, with varying percentages in all examined samples. Moreover, hsa-miR-203 methylation was associated with hypermethylation of hsa-miR-34a, -124a and -196b in NHL but not CLL [36–38].

2.2.7 Metastatic Cancers

Several studies have demonstrated that miRNAs affect the metastatic process by targeting metastasis-related genes [9, 39, 40]. Lujambio et al. [41] investigated whether epigenetic factors determine miRNA expression in metastatic cancer. By treating three lymph node metastatic cell lines with 5-AZA and performing a miRNA microarray analysis, followed by CpG island analysis and bisulfite genomic sequencing, the authors identified three miRNAs that showed cancer-specific CpG island hypermethylation: miR-148a, miR-34b/c, and miR-9 [41]. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited the cells'

Table 2.1 Epigenetically regulated microRNAs

miRNA	Regulation	Cell type	Year of discovery	References
let–7a–3	Hypermethylation	Lung cancer	2007	[64, 65]
miR–1	Hypermethylation	Hepatocellular carcinoma	2008	[69]
miR–1/133a cluster	Hypermethylation	Colorectal cancer	2012	[52]
miR–21	Hypomethylation	Ovarian cancer	2007	[43]
miR–34a	Hypermethylation	Leukemia	2010, 2011	[61-63]
miR–34b/c cluster	Hypermethylation	Metastatic cancers Colorectal cancer	2008 2008	[48, 53]
miR–107	Hypermethylation	Pancreatic cancer	2009	[55]
miR–1224	Hypermethylation	Bladder cancer	2011	[46]
miR–124	Hypermethylation	Leukemia	2010, 2011	[61-63]
miR–124a	Hypermethylation	Colorectal cancer Gastric cancer Leukemia	2007 2009 2009	[47] [54] [57, 58]
miR–126	Hypermethylation	Breast cancer	2008	[39]
miR–127	Hypomethylation, Histone Acetylation	Bladder cancer	2006	[44]
miR–129	Hypermethylation	Gastric cancer	2010	[56]
miR–130b	Hypermethylation	Ovarian cancer	2012	[42]
miR–148a	Histone deacetylation	Breast cancer Metastatic cancers	2012 2008	[45] [40]
miR–152	Hypermethylation	Bladder cancer Breast cancer	2011 2012	[46] [40]
miR–155	Histone deacetylation	Breast cancer	2012	[41]
miR–191	Histone deacetylation	Hepatocellular carcinoma	2011	[71]
miR–200a	Histone acetylation	Hepatocellular carcinoma	2011	[70]
miR–201	Hypomethylation	Ovarian cancer	2007	[43]
miR–203	Hypermethylation	Leukemia	2010, 2011	[61-63]
miR–205	Hypomethylation	Ovarian cancer	2007	[43]
miR–206	Hypermethylation	Breast cancer	2008	[39]
miR–212	Histone methylation Histone acetylation	Lung cancer	2006, 2011	[67, 68]
miR–223	Hypermethylation Histone deacetylation	Leukemia	2007	[60]
miR–224	Histone deacetylation	Hepatocellular carcinoma	2012	[72]
miR–335	Hypermethylation	Breast cancer	2008	[39]
miR–342	Hypermethylation	Colorectal cancer	2008	[49]
Mir–357	Hypermethylation Histone deacetylation	Melanoma	2011	
miR–517	Hypermethylation Histone deacetylation	Hepatocellular carcinoma	2012	[73]
miR–9	Hypermethylation Histone deacetylation	Leukemia Metastatic cancers Colorectal cancer	2011 2008 2009	[59] [50,53]
miR–137	Hypermethylation	Colorectal cancer	2009	[50, 53]
miR–129	Hypermethylation	Colorectal cancer	2009	[50, 53]
miR–941	Hypermethylation	Colon cancer	2011	[51]
miR–1237	Hypermethylation	Colon cancer	2011	[51]
miR–1247	Hypermethylation	Colon cancer	2011	[51]

motility and their metastatic potential in xenograft models and was associated with downregulation of miRNA oncogenic target genes such as c-MYC, E2F3, CDK6, and TGIF2. Finally, promoter hypermethylation of these three miRNAs was significantly associated with metastasis in human malignancies [41].

In summary, an abundance of studies (listed in Table 2.1) show that miRNAs undergo epigenetic regulation, similar to any other PCG. MiRNAs represent an

indirect mechanism through which epigenetics affect the expression of OGs and TSGs and ultimately impact on human carcinogenesis. The complexity of the miRNA-epigenetics relationship is refined by the discovery of a subset of miRNAs, the so-called "epi-miRNAs", that can regulate the expression levels of effectors of the epigenetic machinery.

2.3 MicroRNAs Regulating Effectors of the Epigenetic Machinery

2.3.1 MicroRNAs Regulating DNMTs

The first evidence showing the regulation of DNMTs by miRNAs was provided by Fabbri et al. in 2007 in lung cancer cells. We showed that miR-29 family (29a, 29b and 29c) directly binds to the 3′UTR region of DNMT3A and 3B (*de novo* methyl transferases), two key enzymes involved in DNA methylation [42]. The miR-29 family comprises three isoforms arranged in two clusters: miR-29b-1/miR-29a on chromosome 7q32 and mir-29b-2/miR-29c on chromosome 1q23. MiR-29 family members have been shown to be downregulated in lung cancer [42, 43], and restoration of individual miR-29s induces a marked reduction of DNMT3A and 3B mRNA and protein levels leading to a global DNA hypomethylation, which in turn causes reactivation of epigenetically silenced TSGs such as FHIT and WWOX in cancer cell lines. Interestingly, the same group has also discovered another mechanism of DNMT regulation by miR29s in AML [44]. MiR-29b expression is dysregulated in primary AML blasts and restoration of miR-29b in AML cells results in a marked reduction of DNMT1, 3A, and 3B expression levels, which in turn causes a decrease in overall DNA methylation and re-expression of TSGs such as p15INK4b and ESR1 via promoter DNA hypomethylation. MiR-29b directly targets DNMT3A, and 3B, whereas targeting of DNMT1 is indirect and mediated by SP1, a transactivator of DNMT1. The overexpression of miR-29 induces apoptosis in lung cancer cell lines and reduced tumorigenicity in a xenograft model of lung cancer and AML [42]. These discoveries explored an unknown functional link between microRNAs and aberrant DNA methylation via targeting DNMTs in lung cancer and AML models.

In 2008 Duursma et al. have revealed that miR-148 regulates DNMT3B expression by binding to its coding sequence (CDS) and not to its 3′UTR [45]. In the same year, Benetti et al. discovered a previously unknown DNA methylation mechanism involving the mammalian Dicer-dependent miR-290 cluster that is predicted to target Rbl2 [46, 47]. A substantial down-regulation of the miR-290 cluster was found in Dicer1-null cells compared to wild-type controls [46]. Rbl proteins epigenetically repress DNMT promoters by decreased abundance of AcH3K9 at the promoter regions of the DNMT1, DNMT3A and DNMT3B genes. Over-expression of Rbl2 protein causes decreased expression of DNMTs in Dicer1-null cells, concluding

that increased levels of Rbl2 protein in Dicer1-null cells is responsible for decreased DNMT expression and less DNA methylation in these cells [48]. The miRNA-290 family is highly expressed in pluripotent ES cells and repressed upon differentiation [49]. Altogether, these findings suggest that in the absence of Dicer, downregulation of the miR-290 cluster leads to increased mRNA levels of the miR-290 cluster's target gene Rbl2, whose product in turn inhibits DNMTs expression. Decreased DNMT expression, in part mediated by Rbl2, is leading to a significant hypomethylation of the genome, including the subtelomeric regions, as well as to the appearance of telomeric phenotypes such as increased telomere recombination and increased telomere length [46, 47, 49, 50].

IL-6 has been shown to regulate the activity of DNMT1 and the expression of TSGs by modulation of miR-148a, miR-152 and miR-301, which have a 3′UTR complementarity sequence to DNMT1 [51]. These miRNAs have been found to have decreased levels in IL-6 overexpressing malignant cholangiocytes and in tumor cell xenografts with concomitant decrease in expression of TSGs such as RASSF1A and p16INK4a. Over-expression of miR-148a and miR-152 in cholangiocytes causes decreased DNMT1 protein expression, increased Rassf1a and p16INK4a expression, and reduced cell proliferation [51, 52] providing a link between this inflammation-associated cytokine and oncogenesis in cholangiocarcinoma.

In 2010, Das et al. have explored the role of miR-152 mediated DNMT repression in all-trans-retinoic acid (ATRA) induced neuroblastoma cell line differentiation [53]. ATRA treatment causes downregulation of MYCN, hence leading to overexpression of MYCN repressed miRNAs such as miR-152, miR-26a/b, and miR-125a/b. This downregulates DNMT1 and DNMT3B expression and in turn leads to the demethylation and activation of NOS1, which promotes neural cell differentiation in SK-N-BE cells. Overexpression of miR-152 causes downregulation of DNMT1 that negatively regulates cell invasiveness and anchorage-independent growth, contributing to the differentiated phenotype [53]. These findings illustrate the dynamic nature of the miR mediated epigenome alterations during not only cancer cell proliferation, apoptosis but also during the differentiation process. Also, the expression of miR-152 was normally down-regulated with concurrent increase of DNMT1 expression in HBV induced HCCs [48]. Overexpression of miR-152 resulted in a significant reduction of the expression of DNMT1 via its 3′UTR, which in turn leads to a decrease in global DNA methylation. Moreover, inhibition of miR-152 causes overall DNA hypermethylation and increases promoter DNA methylation of TSGs such as glutathione S-transferase pi 1 (GSTP1) and E-cadherin 1 (CDH1) in HepG2 cells [48].

In 2010, viral miRNAs have been shown to control the epigenetic machinery of host cells through DNMTs [54]. K12-4-5p, a Kaposi sarcoma-associated herpesvirus (KSHV) miRNA was found to regulate the expression of DNMT1, 3A and 3B indirectly, by targeting the expression of Rbl2, a known repressor of DNMT1, 3A and 3B transcription. Ectopic expression of miR-K12-4-5p reduces Rbl2 protein expression and increases DNMT1, -3A, and -3B mRNA levels in 293 cells, thus affecting the overall epigenetic reprogramming of the host cell [54].

Table 2.2 Regulation of DNMTs by microRNAs

miRNA	Target	Cell type	Year of discovery	References
miR–29a,b,c	DNMT3a,3b	Lung cancer	2007, 2009	[1]
miR–29b	DNMT1,3a,3b	AML	2006	[2, 3]
	DNMT3b	Hela cells	2008	[4]
miR–290 cluster	DNMT1,3a,3b	Dicer null cells, Pluripotent ES cells	2008	[5-7]
miR–148a, 152, 301	DNMT1	Cholangiocytes	2010	[9, 10]
K12–4–5p	DNMT1,3a,3b	Viral infection	2010	[11]
miR–152, 26a/b,125a/b	DNMT1, 3b	Neuronal differentiation	2010	[13]
miR–21, 148a	DNMT1	SLE	2011	[14]
miR–342	DNMT1	Colorectal cancer	2010	[15]
miR–152	DNMT1	NiS induced tumorigenesis	2012	[16]
miR–29	DNMT3a,3b	Influenza infection	2012	[17]

More recently, Wang et al. showed that DNMT1 is regulated by miR-342 in CRC [55]. Low expression of miR-342 and high expression of DNMT1 were observed in CRC tissues and cell lines. Downregulation of DNMT1 expression through miR-342 caused reactivation of TSGs such as ADAM23, Hint1, RASSF1A and RECKS through promoter hypomethylation. Restoration of miR-342 resulted in a reduction of DNMT1 expression, reduced cell proliferation, and invasiveness in CRC cells and inhibition of tumor growth and lung metastasis formation in nude mice [55].

Nickel (Ni) compounds are well described human carcinogens. Recently an important regulatory double-negative feedback loop has been discovered between miR-152 and DNMT1 in nickel sulfide (NiS)-transformed human bronchial epithelial (16HBE) cells [56]. Expression of miR-152 was specifically downregulated by promoter hypermethylation, whereas ectopic expression of miR-152 resulted in a remarkable reduction of DNMT1 expression in transformed cells. Interestingly, treatment with 5-AZA or knock down of DNMT1 reversed this process. Further, inhibition of miR-152 expression in 16HBE cells was found to increase DNMT1 expression and DNA methylation. Moreover, ectopic expression of miR-152 caused a significant decrease of cell growth, whereas inhibition of miR-152 reversed this process in 16HBE cells, suggesting the existence of an important functional negative feedback loop between miR-152 and DNMT1, likely to play an important role in NiS induced carcinogenesis [56]. The series of studies showing miRNAs regulating DNMTs is listed in Table 2.2.

Table 2.3 Regulation of HDACs by microRNAs

miRNA	Target	Cell type	Year of discovery	References
miR–140	HDAC4	Muscular differentiation, colon cancer	2006, 2009	[1, 4]
miR–1	HDAC1	Skeletal muscle differentiation	2006	[2]
miR–449a,b	HDAC1	Prostate cancer, lung cancer	2009, 2012	[3, 6]
miR–9	HDAC4,5	Waldenstrom macroglobulinemia	2010	[5]

2.3.2 MicroRNAs Regulating HDACs

The first evidence of miRNA involvement in regulating histone deacetylases (HDACs) expression levels was provided in 2006. Two groups showed first that miR-140 plays an important role in promoting differentiation by suppressing HDAC4 levels, a known co-repressor of Runx2, a transcription factor essential for chondrocyte hypertrophy during skeletogenesis [57]. In the same year it was published that miR-1 promotes differentiation during muscle development by also suppressing HDAC4 [58]. In 2009, Noonan et al. provided a mechanistic insight on the regulation of HDAC1 by miR-449a in prostate cancer [59]. Overexpression of HDAC1 and a low expression of miR-449a were found in prostate cancer cells and tissue samples from patients when compared to their respective controls. MiR-449a binds and targets HDAC1 directly via the 3′UTR transcript. Overexpression of miR-449a resulted in cell-cycle arrest, apoptosis and a senescent-like phenotype by reducing the level of HDAC1 in PC-3 prostate cancer cell line, thus providing a link between miR-449a and HDAC1 that in turn alters the cellular epigenetic program to promoting cell proliferation and survival [59]. MiR-140 has also been shown to be involved in chemoresistance mechanisms by targeting HDAC4 [60]. Inhibition of endogenous miR-140 by locked nucleic acid-modified anti-miRs partially sensitized resistant colon cancer stem-like cells to 5-FU treatment by increasing HDAC4 levels, leading to a G_1 and G_2 phase arrest [60]. Low expression of miR-9 along with high expression levels of HDACs (HDAC4 and 5) were discovered in Waldenstrom macroglobulinemia (WM) [61]. Mir-9 targets HDAC4 and HDAC5 in WM cells. Overexpression of miR-9 causes downregulation of HDAC4, 5, leading to an up-regulation of acetylated-histone-H3 and -H4. This provides evidence that the loss of miR-9 might be responsible for up-regulation of HDAC4 and HDAC5 in WM cells, contributing to the pathogenesis of WM disease [61]. Recently, Jeon et al. showed that miR-449a, b regulate HDAC1 expression by directly targeting its 3′UTR transcript, indicating that this might be one of the reasons for the low miR-449a, b expression and the high expression of HDAC1 in lung cancer [62]. The series of studies showing miRNAs regulating HDACs is listed in Table 2.3.

2.3.3 MicroRNAs Regulating Polycomb Group Proteins (PcG)

The main function of polycomb group proteins (PcG) is the transcriptional repression of various TSGs through chromatin modifications. PcG proteins act together in polycomb repressive complexes (PRC). PRC2 includes the enhancer of zeste 2 (EZH2), the suppressor of zeste 12 (SUZ12), and embryonic ectoderm development (EED). EZH2, a mammalian histone methyltransferase, is the catalytically active component of PRC2 that contributes to the epigenetic silencing of target genes and regulates the survival and metastasis of cancer cells. EZH2 mediates the trimethylation of lysine 27 of histone H3 (H3K27me3) at target gene promoters, leading to the epigenetic silencing of the target genes. This modification of H3 is necessary for the repression of various TSGs.

In 2008, Varambally et al. showed that loss of miR-101 expression with concomitant elevation of EZH2 is most pronounced in metastatic prostate cancer [63]. This reduction in miR-101 expression inversely correlates with increased expression of EZH2 and dysregulation of epigenetic pathways which results in silencing target gene promoters and subsequent cancer progression. Overexpression of miR-101 inhibits the expression and function of EZH2 in cancer cell lines [63]. Inverse correlation between miR-101 and EZH2 was also observed in transitional cell carcinoma [64], glioblastoma [65], gastric cancer [66], and non-small cell lung cancer [67]. In prostate cancer it has been shown that miR-101 can be inhibited by androgen receptor and HIF-1α/HIF-1β [68]. Sander et al. showed that miR-26a was down regulated in a murine lymphoma model and in human Burkitt lymphoma samples [69]. Ectopic expression of miR-26a targets EZH2, inhibits cell proliferation, increases percentage of cells in G_1-phase, and induces apoptosis in Raji and Namalwa cells. Intriguingly, they also found that c-Myc negatively regulates miR-26a, therefore maintaining high EZH2 expression levels in cells and significantly contributing to c-Myc induced tumorigenesis [69]. In 2009, Juan et al. analyzed a regulatory double-negative feedback loop between miR-214 and EZH2 in controlling PcG dependent gene expression during differentiation [70]. PcG proteins suppress the transcription of miR-214 in undifferentiated skeletal muscle cells (SMC). Ectopic expression of miR-214 directly targets EZH2 via its 3′UTR transcript and inhibition of miR-214 rescues this process in differentiating C2C12 cells. Ectopic expression of miR-214 reduces EZH2 expression, increases myogenin expression, and promotes muscle differentiation [70]. EZH2 is also highly expressed in nasopharyngeal carcinoma (NPC) patients and correlates with a higher risk of relapse [71]. Depletion of EZH2 is associated with decreased cell proliferation, induced apoptosis in C666-1 cells and delayed tumor growth in SCID mice. In this model three miRNAs (namely miR-26a, miR-98, and 101), whose expression is consistently downregulated in human NPC specimens when compared to normal nasopharyngeal epithelial tissue samples, have been shown to directly target EZH2 [71]. Recently, there has been an extensive series of studies unraveling a central role of miR-101 in the regulation of EZH2 in several types of cancer. In hepatoma tissues, it was shown that miR-101 and miR-29c are downregulated, but their

Table 2.4 Regulation of EZH2 by microRNAs

miRNA	Target	Cell type	Year of discovery	References
miR–101	EZH2	Prostate cancer	2008, 2010	[24, 32]
		Bladder transitional cell carcinoma	2009	[27]
		Glioblastoma	2010	[30]
		Gastric cancer	2010	[31]
		Hepatocellular carcinoma	2010	[33]
		Nasopharyngeal carcinoma	2010	[29]
		Angiogenesis	2011	[34]
		NSCLC	2011	[35]
miR–26a	EZH2	Muscle differentiation	2008	[25, 26]
		Burkitt lymphoma		
		Nasopharyngeal carcinoma	2010	[29]
miR–214	EZH2	Skeletal muscle differentiation	2009	[28]
miR–98	EZH2	Nasopharyngeal carcinoma	2010	[29]
miR–29c	EZH2	Hepatocellular carcinoma	2010	[33]

expression can be restored (leading to reduced levels of EZH2, EED and H3K27me3 proteins) after treatment with TPA (12-O-tetradecanoylphorbol 13-acetate), which is Protein Kinase C (PKC) and ERK pathway dependent in HepG2 cells [72]. Also, Smiths et al. have established a pro-angiogenic effect of miRNA-101 working together with EZH2 and VEGF during the process of angiogenesis [73]. The group analyzed the expression of miR-101 in endothelial cells derived from glioma patients and found to it be low. VEGF downregulates the expression levels of miR-101 resulting in increased protein expression of EZH2, induces elongation of endothelial cells leading to a pro-angiogenic response. Transfection with pre-miR-101, or EZH2 siRNA, or treatments with DZNep, a small inhibitor of EZH2 methyltransferase activity, reverses this process in HBMVECs controls, providing a network between VEGF/miR-101/EZH2 proteins towards pro-angiogenic response in endothelial cells [73].

Overall, an increasing number of studies has identified a central role for miRNAs as modulators of key effectors of the epigenetic machinery, revealing a more complex layer of reciprocal regulation between "traditional" epigenetic effectors (such as DNMTs. HDACs, PcG) and ncRNAs. The series of studies showing miRNAs regulating PcGs is listed in Table 2.4.

2.4 Conclusion

MiRNAs play a central and pivotal role in the regulation of gene expression. The series of studies covered in this chapter clearly indicate that while these small ncRNAs are kept under a rigorous epigenetic control in several different types of tumors, they can actually also affect the expression of other epigenetically

regulated PCGs by targeting key effectors of the epigenetic machinery. Therefore, miRNAs interpose their action between DNMTs, HDACs, PcGs and their epigenetic target PCG. Intriguingly, the world of ncRNAs is being more and more extensively studied and is being populated by an increasing number of biologic transcripts. Among them, the transcribed ultraconserved regions (T-UCRs) also play an important role in human carcinogenesis [5]. Noteworthy, it has been shown that miRNAs can regulate the expression of T-UCRs, suggesting an additional layer of complexity in gene expression regulation, involving two different groups of ncRNAs [5]. Moreover, it was demonstrated that certain miRNAs directly target transcription factors regulating the expression of other miRNAs. By doing this, it has been observed that one miRNA ultimately affects the expression levels of another miRNA [6]. This increasing complexity of interactions should not scare. Indeed, it can be safely stated that cancer is probably the most complex genetic disease. A better comprehension of such a complexity, while a little bit disorienting at first, it is actually the necessary background to fully understand the whole picture of the epigenetic regulation in human malignancies. Such a knowledge represents the necessary platform to build new treatments based on the biologic rationale provided by these discoveries and ultimately to offer new therapeutic options to cancer patients.

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Chapter 3
Epigenetic Regulation of EZH2 and Its Targeted MicroRNAs

Sandy Leung-Kuen Au, Irene Oi-Lin Ng, and Chun-Ming Wong

Abstract Polycomb group (PcG) proteins are transcriptional repressors which function to silence expressions of developmental and differentiation genes in eukaryotic cells. PcG proteins assemble into complexes termed Polycomb Repressive Complex (PRC) 1 and 2, and they elicit a cascade of epigenetic silencing events starting from trimethylation of the 27th lysine residue on histone H3 by the core PRC2 protein Enhancer of Zeste Homolog 2 (EZH2). In human cancers, PcG-mediated epigenetic silencing activity is increased as a result of upregulation of EZH2 and other PcG proteins. Consequentially, EZH2 is implicated in cancer development through epigenetic repression of tumor suppressor genes. MicroRNAs (miRNAs) are small, endogenously produced non-coding RNAs which function to negatively regulate the expression of their target mRNAs. MiRNA regulation is widespread and virtually over all cellular processes. In recent years, miRNAs have emerged as critical mediators in cancer pathogenesis. Remarkably, EZH2 can epigenetically silence miRNAs, while miRNAs also exert negative control over EZH2 expression, establishing a self-regulatory loop to reinforce their cancer specific roles. In this chapter, we review the current understanding of EZH2 and its regulated miRNAs in malignancies.

Keywords Cancer epigenetics • Histone modifications • Polycomb group proteins • Epigenetic-microRNA regulatory circuit

S.L.-K. Au • I.O.-L. Ng (✉) • C.-M. Wong (✉)
State Key Laboratory for Liver Research and Department of Pathology,
LKS Faculty of Medicine, The University of Hong Kong,
University Pathology Building, Queen Mary Hospital, Pokfulam, Hong Kong
e-mail: iolng@hkucc.hku.hk; jackwong@pathology.hku.hk

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3.1 Introduction

Polycomb group (PcG) proteins are transcriptional repressors tightly regulating development and differentiation-associated genes throughout embryonic stage to adulthood. These proteins also play pivotal roles in cancer development [1, 2]. At the molecular level, PcG proteins assemble into complexes to remodel chromatin structure by establishing and propagating histone post-translational modifications [3, 4]. PcG activity is elevated in diseases such as cancer, and is causal to aberrant epigenetic silencing of tumor suppressor genes [5, 6]. Endogenous non-coding RNAs (ncRNAs) are ubiquitously transcribed in the genome and their functions are beginning to be elucidated. MicroRNAs (miRNAs) are small non-coding RNAs of size 18–25 nucleotides in length [7]. In the human genome, over 2,000 mature miRNA species have been annotated thus far (miRBase Release 19), highlighting the diversity of miRNA regulation in cellular processes. Functioning as post-transcriptional negative regulators, miRNAs repress gene expression through sequence-specific interactions with the 3′ untranslated regions (UTR) of target mRNAs [7]. Interestingly, miRNAs and epigenetic circuitry are robustly connected through reciprocal regulation of one other. This self-regulatory loop exists when cells undergo normal differentiation or go astray to become oncogenic.

In this chapter, we will first recapitulate the function of PcG proteins in epigenetic programming of gene expressions. Specifically, we will focus our discussion on the histone methyltransferase Enhancer of Zeste Homolog 2 (EZH2) and its epigenetic activity. Next, we will review the biology of miRNAs and provide several examples of cancer-specific miRNAs. Lastly, miRNA-mediated regulation of EZH2 and conversely, the importance of EZH2-silenced miRNAs in tumorigenesis will be covered. A better understanding of the PcG-miRNA regulatory axis will surely benefit development of new diagnostic markers and therapeutic targets for cancer treatment.

3.2 Polycomb Mediated Epigenetic Silencing Machinery

3.2.1 Polycomb Group Proteins in Mammalian System

PcG proteins are chromatin modifiers that are evolutionarily conversed in plants and animals. They were initially identified in *Drosophila melanogaster* that functioned to stably repress HOX genes, which are transcription factors specifying spatial and temporal expressions of body segment during development. In male flies, mutations of different PcG proteins cause misexpression of HOX genes, giving rise to a phenotype with transformation of body segment and formation of extra sex comb (also termed as Polycomb) on the legs [8]. In the mammalian system, different homologs of PcG proteins exist and they function collaboratively to establish a chromatin structure through histone post-translational modifications for stable transcriptional repression of developmental genes [9] and X-chromosome inactivation [10].

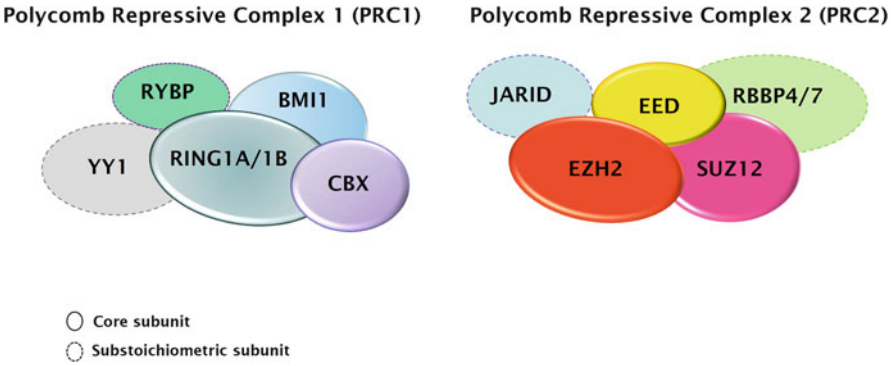

Fig. 3.1 Schematic diagram illustrating the assembly of mammalian Polycomb group (PcG) proteins into functional Polycomb Repressive Complex (PRC) 1 and 2. The PcG-mediated epigenetic machinery based on PRC2 to establish trimethylation on histone H3 lysine 27 (H3K27me3), and subsequent recruitment of PRC1 to compact the chromatin loci for physical hindering of gene transcription. PRC1 core protein subunits include RINGs, BMI1 and CBXs that specialize in histone H2A ubiquitylation and chromatin binding via H3K27me3 recognition. PRC2 core protein subunits include EZH2, EED and SUZ12 that catalyze H3K27me3 on target gene loci. Other substoichiometric components of the complexes are also illustrated

At the molecular level, PcG proteins assemble into multimeric complex termed as Polycomb Repressive Complexes (PRCs). Two groups of PRCs are more well-characterized, namely PRC2 and PRC1 (Fig. 3.1). PRC2 is composed of Enhancer of Zeste Homolog 2 (EZH2) for catalyzing trimethylation on the 27th lysine reside on histone H3 (H3K27me3) [3], Suppressor of Zeste 12 (SUZ12) [11] and Embryonic Ectoderm Development (EED) [12] as other core structural subunits. Components of PRC1 are more diverse, including chromobox (CBX), B lymphoma Mo-MLV insertion region 1 (BMI1) and the ubiquitin E3 ligase RING1A/1B as core subunits [13]. Series of biochemical studies have further unraveled multiple isoforms of core and non-core PRC proteins that assemble into non-canonical PRCs in different developmental stage and under cellular context [14, 15]. However, their definitive roles, especially in the cancer pathological scenario, remain largely elusive.

3.2.2 PRC2-Mediated H3K27me3 for Epigenetic Repression

In the classic model of PcG-mediated epigenetic repression (Fig. 3.2), PRC2 imposes H3K27me3 through the histone methyltransferase activity of EZH2 at its target loci. PRC1 recognizes the H3K27me3 through the chromodomain of CBX proteins and direct the complex to the H3K27me3-bearing loci [3]. Repression of gene transcription is achieved by a number of ways, for example by further ubiquitylation on the 119th lysine residue on histone H2A to compact the chromatin [4], via direct hindering of RNA polymerase II from starting the transcription [16], and in some

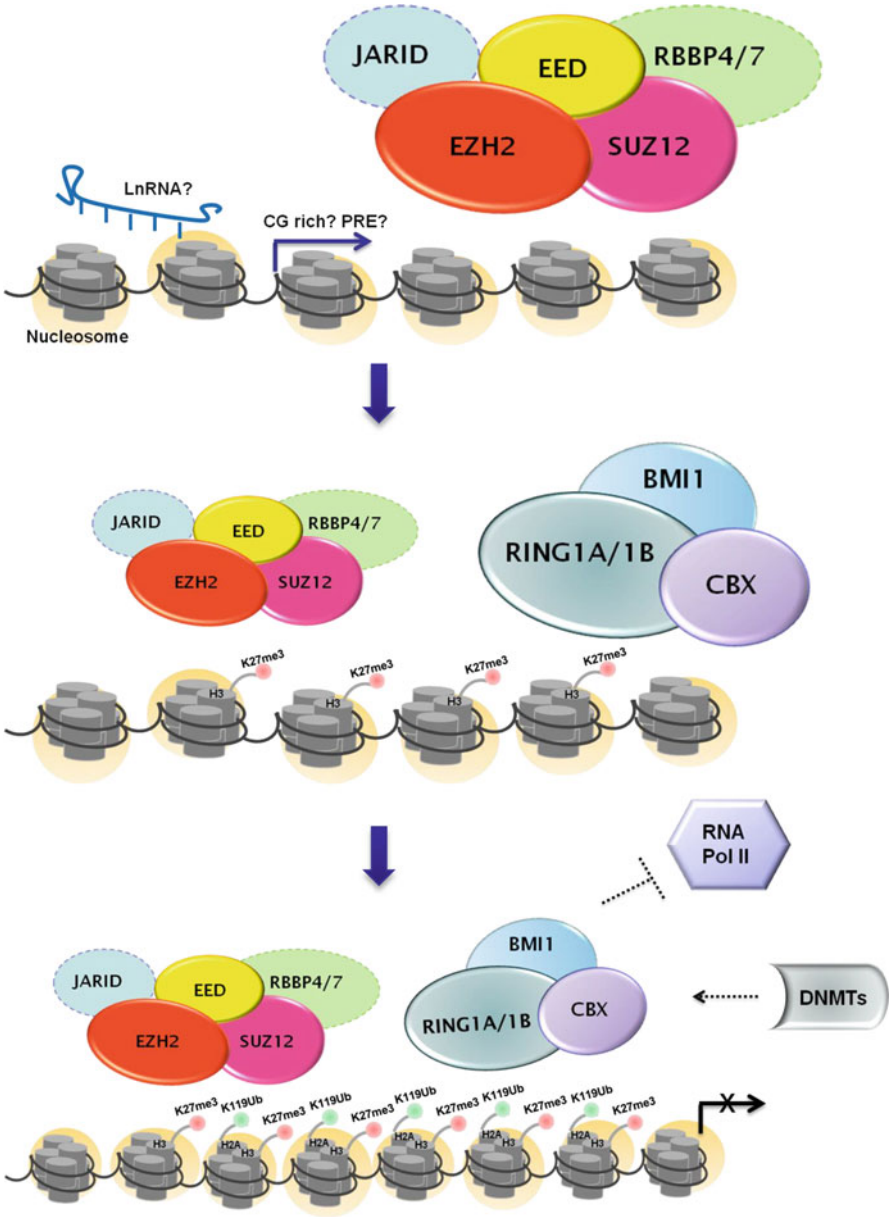

Fig. 3.2 Coordinated epigenetic silencing machinery of PRCs. In the mammalian systems, it remains uncertain on how PRC2 is initially recruited. The involvement of long non-coding RNA (lnRNA), CG rich sequences upstream of transcription start site, and consensus sequence specific binding proteins (e.g. JARID) are suggested to direct PRC2 to target gene loci. Polycomb repressive elements (PREs), which are DNA elements defined from PcG targets in *Drosophila*, are still largely elusive in mammalian genome. To repress gene transcription, EZH2 of PRC2 first catalyzes H3K27me3. This histone modification is further recognized by chromodomain of CBX in PRC1, and RING subsequently monoubiquitylates the 119th lysine on histone H2A (H2AK119Ub). PRC1 inhibits gene transcription by chromatin compaction or physically hindering RNA polymerase II. In some cases, DNA methyltransferases (DNMTs) may also be recruited to methylate the loci for stable and irreversible repression

cases recruitment of DNA methyltransferases to further methylate DNA [17]. Of note, in this classic model, the molecular basis of initial recruitment of PRC2 to target loci is incompletely understood in the mammalian system. In *Drosophila*, PcG target genes are well-characterized by the presence of Polycomb response elements (PREs), which is specific DNA sequences recognized by the pleiohomeotic (PHO)-containing PRC [18]. In mammals, precise PREs remain obscure. Large GC-rich sequence elements lacking transcriptional activation signals may function as general PRC2 recruitment elements [19]. It is also suggested that the mammalian homolog of PHO, the Yin Yang 1 (YY1) zinc-finger transcription factor, may play some role in directing PRC2 to target loci bearing YY1 consensus motif [20]. In addition, Jumonji-and ARID-domain-containing protein (JARID2) is also suggested to be associated with PRC2 and required for genome-wide localization of the complex [21]. Recently, the involvement of long non-coding RNAs (lncRNA) in recruiting PRC2 to target loci has been demonstrated. LncRNAs such as XIST is implicated in X-chromosome inactivation [10], KCNQ1OT1 can mediate PRC2 spreading *in cis* to maintain imprinted expression of KCNQ1 domain [22], and HOTAIR can promote PRC2 binding *in trans* to repress HOXD expression [23]. Further study is warranted to understand what the determinants are and how they participate in the initial global definition of PcG targets and PRC recruitment.

3.2.3 Polycomb Target Genes Are Developmental and Cell-Fate Regulators

Genome-wide mapping of PcG targets in embryonic stem (ES) cells and differentiated somatic cells suggest that throughout development and adulthood, PcG-regulated genes are essential for proper cell differentiation, retaining stem-cell plasticity and maintaining cell identity. In ES cells, PRC2 targets are conservatively estimated to represent at least 10 % of the genes. These targets include transcription factors of the Pax, Lhx and Dlx gene families and also components of signaling pathways like Wnt, TGFβ, FGF and BMP, which require repression until differentiation and cell lineage commitment is promoted [9, 24, 25]. PcG regulation in maintaining ES cell pluripotency is linked to repressing stem cell transcription factors OCT4, SOX2 and Nanog [9].

One prominent chromatin feature of PcG targets is the “bivalent” chromatin domain consisting of the transcription repressive H3K27me3 modification and di/trimethylation of the 4th lysine residue on histone H3 (H3K4me2/me3) as an activating modification. With this bivalent chromatin feature, PcG targets are held in a “ready-to-transcribe” status until appropriate transcription signal is received [26, 27]. Recently, the involvement of PcG epigenetic marking on genes is intricately implicated in *de novo* cancer specific DNA methylation [28, 29], suggesting that PcG-mediated epigenetic silencing plays an indispensable role in cancer development.

3.3 Core of PcG-Mediated Epigenetic Silencing: Enhancer of Zeste Homolog 2

Owing to the indispensable roles of PcG proteins in cellular development, deregulation of them is impeccably linked to cancer development. Accumulating evidence has indicated the aberrant expressions of PRC2 and PRC1 proteins in human malignancies (Table 3.1), and EZH2 is among the most frequently up-regulated PRC2 proteins [30–32]. The central of PcG-mediated epigenetic repression relies on the establishment of H3K27me3 by EZH2. In the subsequent sections, we will focus the discussion on EZH2 and review its functional consequence on miRNA deregulation.

3.3.1 Identification of EZH2

In *Drosophila*, maintenance of segment identity gene repression is controlled by at least 11 PcG genes, and the Enhancer of Zeste (EZ) is one of those. EZ was first identified as dominant gain-of-function modifiers of the zeste-white eye color, in which EZ gene activity was required for repression of white eye color pigment through interaction with the transcription factor zeste. Mutant EZ alleles also produced homeotic transformations of body segment and reduction of EZ resulted in expression of some antennapedia and bi-thorax body segment genes, an observation consistent to alterations in other PcG genes [33].

Table 3.1 Aberrant expressions of PcG proteins in human cancers

	PcG protein	Expression in cancer	Cancer Type
PRC2	EZH2	Increased	B cell non-Hodgkin lymphoma, bladder cancer, breast cancer, colon cancer, glioblastoma, liver cancer, lung cancer, melanomas, prostate cancer
	SUZ12	Increased	Breast cancer, colon cancer, liver cancer, lung cancer, melanomas
	EED	Increased	Liver cancer
PRC1	BMI1	Increased	Acute myeloid leukemia, B cell non-Hodgkin lymphoma, breast cancer, leukemia, liver cancer, lung cancer, neuroblastoma
	RING1B	Increased	Bladder cancer, breast cancer, colon cancer, kidney cancer, liver cancer, lung cancer, lymphoma, prostate cancer
	CBX7	Increased	Follicular lymphoma
	YY1	Increased	B non-Hodgkin lymphoma, breast cancer, liver cancer
	RYBP	Decreased	Liver cancer, prostate cancer

PRC2 and PRC1 proteins are frequently deregulated in human cancers. Expressions of many of them are found to be up-regulated, indicating an increase of PcG-mediated epigenetic silencing activity during cancer development

EZH2 is one of the human homologs of EZ which was identified from screening of human B-cell-specific cDNA library using the conserved carboxy-terminal of Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain of *Drosophila* EZ [34]. In year 1996, Chen et al. first reported the mapping of EZH2 gene to chromosome 21 q22.2 by using exon trapping in cosmid DNA from a human chromosome 21 specific library [35]. The actual genomic localization of EZH2 gene was only corrected in year 2000 by Cardoso et al. who indicated that the gene was indeed mapped to chromosome 7q35-q36, and on chromosome 21 was a pseudogene of EZH2 which could not be expressed [36].

3.3.2 Tissue and Cell Type Expression of EZH2

EZH2 is ubiquitously expressed in diverse tissues including the testis, heart, pancreas, adrenal gland and liver as detected by Northern blotting and immunohistochemistry [37]. EZH2 expression varies during normal development and differentiation of cells. For example, EZH2 is abundantly expressed during early embryogenesis but restricted to fetal hematopoietic site in late development [38]. In other normal physiological processes, EZH2 expression may also be altered. For example, EZH2 is expressed in actively dividing but not resting peripheral T cells and is implicated in T cell differentiation [39]. Loss of EZH2 expression in wound-edge cells is observed during murine skin repair with concomitant up-regulation of wound repairing genes [40]. In human ES cells, EZH2 expression in association with other PcG proteins diminishes upon cell differentiation [41].

3.3.3 Cellular Localization of EZH2

EZH2 is predominantly localized within the nuclei of cells. On regions of pericentric heterochromatin, EZH2 is in association with other PcG proteins forming PcG bodies [42]. Endogenously expressed EZH2 can be colocalized with PCNA and CAF1 at site of DNA replication during mitosis [43]. EZH2 can also be specifically recruited to and localized with the X chromosome during its inactivation in trophoblast stem cells and ES cells [10]. Interestingly, cytoplasmic localization at the site of actin polymerization of EZH2 in association with the guanine nucleotide exchange factor Vav1 is also reported in *ex vivo* thymocytes, primary mouse embryonic fibroblasts, the human embryonic kidney (HEK)-293 cells [44] and benign prostate epithelial cells [45].

3.3.4 Molecular Structure and Functional Domains of EZH2

Full length form of EZH2 protein is encoded by 752 amino acid residues with molecular size of 98 kDa. EZH2 protein consists of six main structural domains (Fig. 3.3).

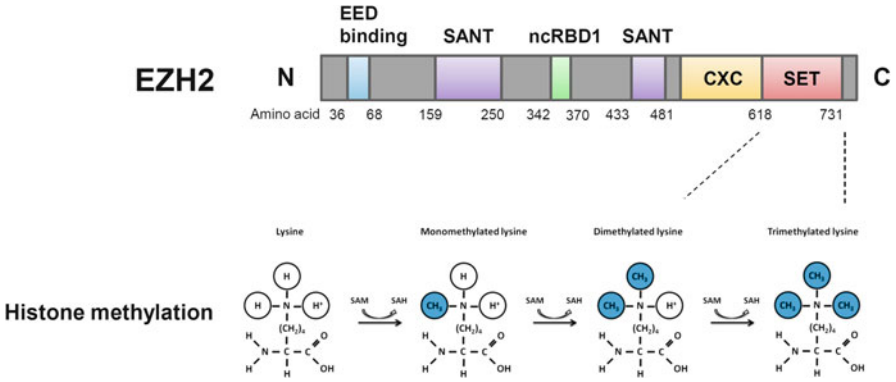

Fig. 3.3 Schematic representation of EZH2 molecular structure (upper panel) **and histone methylation process** (bottom panel). *Upper panel*: EZH2 consists of six main structural domains. EED binding domain is required for interaction with EED. SANT domains are required for DNA binding. Non-coding RNA binding domain (ncRBD1) is responsible for interaction with ncRNAs such as HOTAIR and Xist RepA. Cystine-rich CXC domain accommodates zinc ions for EZH2's enzymatic activity. SET domain is the catalytic domain for histone methyltransferase activity. *Bottom panel*: Histone methylation by SET domain is proposed to be a step-wise process. Each round of methylation transfers one methyl group from the methyl donor SAM, eventually generating a trimethylated lysine. SAM: S-adenosyl-L-methionine; SAH: S-adenosylhomocysteine

The catalytic domain for histone methyltransferase activity is the SET domain localized on the carboxyl-terminal. The SET domain has a unique pseudoknot structure formed by juxtaposition of two conserved peptide motifs within the domain, which allows the lysine substrate and the methyl donor to bind to opposite sides of the domain. The detailed process of lysine methylation on histone is still elusive, but one possible mechanism includes a stepwise process involving the transfer of a methyl group from the methyl donor S-adenosyl-L-methionine (SAM/AdoMet) to the amino group of lysine residue to form the mono, di and eventually trimethylation [46]. In this regard, the SET domain therefore has unique arrangement of substrate-binding sites which can permit multiple rounds of lysine methylation [47]. The initial identification of EZH2 histone methyltransferase activity from *Drosophila* EZH2-containing complex showed its preference toward both H3K9 [48] and H3K27, but H3K27 was the major site for the methylation [3]. Adjacent to SET domain is the cystine-rich CXC domain, which is required to structurally couple zinc ions for complete enzymatic activity [47]. For robust histone methyltransferase activity, EZH2 binds to other non-catalytic PRC2 subunits, including EED via the EED/WD binding domain [49] and SUZ12 before the CXC and SET domain [50]. The SANT domains are required for DNA binding. Recently, a new domain termed non-coding RNA binding domain (ncRBD1) has been identified that may be responsible for EZH2 interaction with ncRNA such as HOTAIR and Xist RepA RNA [51].

3.3.5 Phosphorylation and Transcriptional Regulation of EZH2

In somatic cells, activity of EZH2 can be regulated by kinases. EZH2 protein contains a number of phospho-serine and phospho-threonine residues, as revealed by phosphoproteomic analyses. Phosphorylation of EZH2 at certain critical residues alters its function in both positive and negative manners. In proliferating cells where EZH2 expression and cyclin-dependent kinases (CDKs) activities are high, phosphorylation of EZH2 by CDKs is functionally critical. Threonine (Thr) 345 phosphorylation of EZH2 by cyclin-dependent kinase 1 (CDK1) is shown to enhance the binding of ncRNAs HOTAIR and RepA during G2/M phase of cell cycle [51]. Additionally, CDK1 and CDK2 can also phosphorylate EZH2 at Thr 350 in the evolutionarily conserved K(R)S(T)PXK(R) motif. The phosphorylation is important for EZH2 recruitment and maintenance of H3K27me3 levels at EZH2-target loci, including HOXA9 [52]. On the other hand, CDK1 phosphorylation of EZH2 at Thr 487 is shown to inhibit EZH2 activity by disrupting its binding with SUZ12 and EED. CDK1 activation in human mesenchymal stem cells promotes differentiation into osteoblasts via Thr 487 phosphorylation of EZH2, which leads to removal of repressive marking on differentiation promoting genes [53]. In addition to CDK, the phosphoinositide 3-kinase–Akt (PI3K-Akt) signaling pathway can negatively regulate EZH2 function. Phosphorylation of EZH2 on Serine (Ser) 21 by Akt reduced EZH2 substrate affinity towards histone H3 and subsequently decreased H3K27me3 level with derepression of EZH2 targets [54].

Transcriptional regulation of EZH2 involves the retinoblastoma protein (pRB)-E2F pathway which is shown to induce EZH2 expression in non-transformed human fibroblasts. EZH2 expression accumulates in actively proliferating cells at the G1/S phase and the induction of its expression is transactivated by binding of E2F to upstream of EZH2 promoter at two potential E2F binding sites. In transformed cells with dysfunctional pRB, E2F can up-regulate EZH2 expression, thereby conferring a growth advantage in the PRC2-complex dependent manner [55]. The microenvironment of solid tumors lacks enough oxygen supply (termed as hypoxia) and it is increasingly recognized as an important element in driving cancer aggressiveness and chemoresistance [56]. Under hypoxic condition, the transcription factor hypoxia-inducible factor-1α (HIF1α) is stabilized to mediate expression of hypoxia-related genes [57]. Interestingly, EZH2 promoter region contains a consensus sequence of HIF response element (NCGTG) which is recognized by HIF1α under hypoxic condition to drive EZH2 transcription in breast tumor initiating cells [58].

3.3.6 Frequent Up-Regulation of EZH2 in Cancers

Disruption of epigenetic balance as a result of aberrant expressions of epigenetic regulators is closely associated with cancer development. EZH2 is often found to be up-regulated both transcriptionally and translationally in a variety of human solid and hematopoietic cancers, and strongly correlates with a poor prognosis [30, 32, 59].

Table 3.2 EZH2 epigenetically silences multiple tumor and metastasis suppressor genes to contribute to cancer development

Gene	Cancer	Function	References
$p16^{INK4A}$-$p14^{ARF}$	Acute myeloid leukemia	Escape senescence, apoptosis and cell cycle arrest	Paul et al. [60]
$p57^{KIP2}$(CDKN1C)	Breast cancer		Yang et al. [61]
p21(CDKN1A)	Melanoma		Fan et al. [62]
RUNX3	Gastric, breast, prostate, colon, and pancreatic cancer cell lines	Promote proliferation	Fujii et al. [63]
BRCA1	ER-negative breast cancers	Promote proliferation	Gonzalez et al. [64]
BMPR1B	Glioblastoma-initiating cells	Inhibit normal differentiation and promote proliferation	Lee et al. [65]
AXIN2, NKD1, PPP2R2B, PRICKLE1, and SFRP5	HCC	Promote proliferation	Cheng et al. [66]
E-cadherin	Gastric and breast cancers	Promote EMT	Cao et al. [5]
DAB2IP	Prostate cancer	Migration and invasion	Min et al. [67]
Rap1GAP	Head and neck squamous cell carcinoma	Invasion	Banerjee et al. [68]
SLIT2	Prostate cancer	Invasion	Yu et al. [69]
PSP94	Prostate cancer	Transformation and cell invasion	Beke et al. [70]
ABRB2	Prostate cancer	Transformation and cell invasion	Yu et al. [71]

In human cancers, EZH2 functions as a general oncogene through epigenetic repression of cell proliferation-control and anti-migration/invasion genes

We have also previously reported that around 70 % of hepatocellular carcinoma (HCC) have EZH2 overexpression at both mRNA and protein levels. More strikingly, EZH2 up-regulation is associated with certain metastatic HCC features, including the presence of venous invasion, direct liver invasion and absence of tumor encapsulation, suggesting that aberrant expression of EZH2 is implicated in HCC aggressiveness [31].

The oncogenic role of EZH2 in cancers is attributed to its epigenetic function in transcriptional repression of cell cycle regulators to promote proliferation or of tumor and metastasis suppressors to promote invasion and metastasis (Table 3.2). Recently, the first recurrent somatic mutation of EZH2 at the 641st tyrosine residue (Y641) has been reported in 21.7 % of GCB subtype of diffuse large B-cell lymphoma and 7.2 % of follicular lymphoma [72]. This mutation is shown to be of gain-of-function type and lead to increase of H3K27me3 in B-cell lymphoma cell lines as well as enhanced enzymatic activity *in vitro* [73]. Subsequent studies continue to discover EZH2 somatic mutations in other residues (e.g. A677) that also alter EZH2 substrate preference [74].

3.4 The Small But Significant Non-coding RNA: MicroRNA

MiRNAs represent one of the best studied classes of endogenous single-stranded small ncRNAs. In 1993, Ambros et al. [75] and Ruvkun et al. [76] first described the miRNA lin-4 as post-transcriptional modulator of lin-14, a developmentally

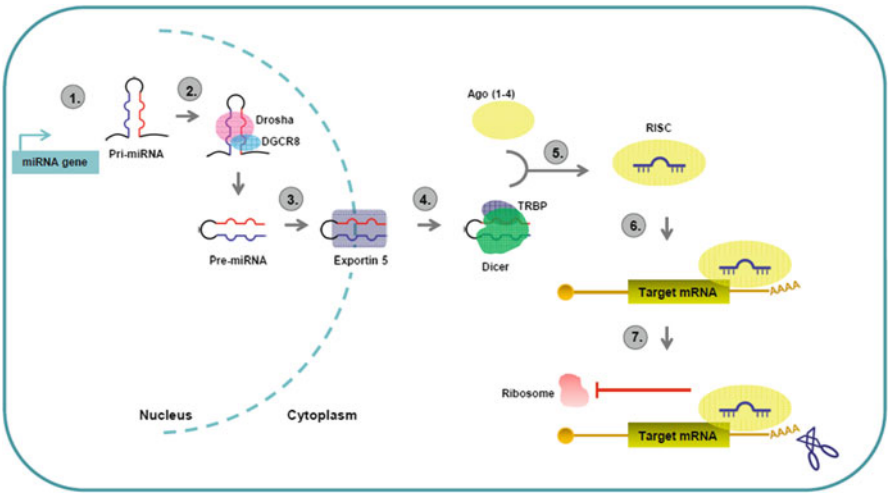

Fig. 3.4 Biogenesis of miRNAs and their repression of target mRNA. Inside the nucleus, miRNA gene, similar to protein-coding genes, is also transcribed by RNA polymerase II into primary-miRNA (pri-miRNA) transcript (*1*). Pri-miRNA is then processed by Drosha/DGCR8 complex to generate precursor-miRNA (pre-miRNA) (*2*). Pre-miRNA is exported via Exportin 5 into the cytoplasm (*3*) and cleaved by Dicer to form mature miRNA duplex (*4*). In the RNA-induced silencing complex (RISC), mature miRNA is loaded into Ago protein (*5*) for target mRNA recognition at the 3′ UTR (*6*). Expression of putative mRNA target is inhibited by various mechanisms, such as blocking initiation or elongation of translation and deadenylation of mRNA (*7*)

important gene in *Caenorhabditis elegans.* Since then, the involvement of miRNAs to negatively regulate their target mRNA expression is well recognized. MiRNAs are usually of 18–25 nucleotides in length which are ubiquitously transcribed in the prokaryotic and eukaryotic genomes (67). According to the miRNA database (http://www.mirbase.org/), over 2,000 mature miRNAs have been identified in the human genome thus far and they are estimated to target more than 30 % of protein-coding genes [77, 78].

3.4.1 Biogenesis and Targeting Mechanism of miRNAs

MiRNA biogenesis involves a cascade of processes occurring inside the nucleus and cytoplasm (Fig. 3.4). The first step in miRNA biogenesis is transcription of miRNA loci. MiRNA genes are found in all chromosomes except the Y chromosome. Genomic locations of miRNA genes can be broadly classified into three types: intronic miRNA in protein-coding transcription unit (61 %); intronic miRNA in non-coding transcription unit (18 %) and intragenic miRNA as a non-coding transcription unit (20 %). MiRNA genes may share the promoters of their host transcripts or have their own promoters, which are still unclearly defined [78]. Most miRNA genes are transcribed by RNA polymerase II into primary miRNA

(pri-miRNA) transcripts of several kilobases in length and with stem-loop structures. The pri-miRNA is cropped by the Microprocessor, which is a large complex consisting of Drosha and DGCR8, in the nucleus to generate hairpin-like precursor miRNAs (pre-miRNAs) of about 65 nucleotides long [79]. The pre-miRNAs are then exported into the cytoplasm through the Ran-dependent nuclear transport receptor exportin 5 (Exp5) [80]. In the cytoplasm, the pre-miRNAs are further cleaved by RNase III Dicer near the terminal loop to produce miRNA duplexes of about 22 nucleotides long [79]. The miRNA duplexes are loaded into Argonaute (Ago) protein, which is in complex with Dicer, TRBP and/or PACT to assemble the miRNA-containing RNA induced silencing complex (miRISC). In the Ago protein, one strand of the miRNA duplex remains as the guide strand or mature miRNA, whereas the other strand is degraded. The strand selection may depend on the thermodynamic stability of pre-miRNAs and in some cases, both strands can also form mature miRNAs [7].

Targeting of mRNA relies on the mature miRNA loaded in the Ago proteins. Ago proteins mediate endonucleolytic cleavage of target mRNA, physically repress translation or initiate deadenylation of mRNA to cause its decay. In the human genomes, there are four members of Ago proteins (Ago1-4) sharing functional redundancy in miRNA repression [81]. The recognition of target mRNA at its 3′UTR depends primarily on its interaction with miRNA at the "seed" region (the second to eighth nucleotides from the 5′ end of the miRNA). On the 3′UTR of target mRNA, usually multiple binding sites for the same or different miRNA can be found which are required for more effective repression [82].

3.4.2 Differentiation and Lineage-Specific miRNAs Are Repressed by PcG Proteins

MiRNA expression is highly cell-type and cellular-process-specific, suggesting that developmental and signaling control of miRNA expression is important to maintain appropriate miRNA functions in cells [78]. Multiple lines of evidence indicate that miRNAs are tightly involved in early mammalian development. In ES cells and embryonic tissues, a subset of miRNAs is preferentially expressed when compared to mature somatic cells and adult tissues [83]. When ES cells are deficient in miRNA-processing enzymes, they exhibit defects in differentiation and proliferation [84]. Dicer-deficient mice are embryonic lethal, which may be partly attributed to a failure to process developmentally important miRNAs [85]. Furthermore, accumulating studies have demonstrated that specific miRNAs participate in controlling cellular differentiation through regulation of their target genes. For example, miR-214 targeting EZH2 is involved in skeletal muscle cell differentiation [86]; and miR-290 family targeting cyclin E/Cdk2 pathway ensures that ES cells undergo proper cell-cycle to proliferate [87].

To explore how miRNAs genes are regulated by core transcriptional regulatory circuitry in ES cells, Marson et al. undertook the study using high-resolution ChIP-sequencing, systematic identification of miRNA promoters, and quantitative

sequencing of short transcripts in multiple cell types to elucidate the coordinated regulation of ES-specific miRNAs [88]. Interestingly, PcG proteins are found to co-occupy lineage-specific miRNAs that are silenced in ES cells. The promoters of a subset of Oct4/Sox2/Nanog/Tcf3-occupied miRNA genes, such as miR-9, miR-124, miR-155, miR-375, miR-615 and miR-708 are occupied by the PRC2 component SUZ12 and enriched with the repressive histone modification H3K27me3 [88]. Upon cellular development, PcG-silencing is lost and these miRNAs are expressed to confer lineage commitment. For example, miR-9 expression is elevated in neural precursor cells but not in embryonic fibroblasts to promote neural differentiation. Taken together, the study implies that PcG repression is impeccably linked to silencing of cell-fate determinant miRNAs during mammalian development.

During cellular differentiation, miRNA and PcG repression machinery may establish feedback regulatory loop to sustain the regulation. Juan et al. have demonstrated a double-negative feedback regulation between EZH2 and miR-214 that is important for skeletal muscle cell (SMC) differentiation [86]. In undifferentiated myoblasts, miR-214 locus is occupied by SUZ12, EED and BMI1 for PcG-mediated repression. When cellular differentiation begins, EZH2 expression is lost and accompanied by an increase in MyoD and myogenin, two key developmental regulators for myogenesis. Interestingly, loss of EZH2 and recruitment of MyoD and myogenin on miR-214 locus drives its expression, which further negatively feeds back on PcG machinery by targeting EZH2 3′UTR to accelerate the differentiation process [86]. The identified EZH2/miR-214 regulatory loop underscores the importance of a robust regulation between PcG proteins and miRNAs that is indispensible for cellular differentiation.

3.5 Deregulation of miRNA in Cancers

Over the past decade, it has become clear that miRNA expressions are dramatically deregulated during cancer development. The first evidence of alterations of miRNA genes and dysregulation of miRNA gene expression in human cancer arose from the studies by Carlo M. Croce's team on chronic lymphocytic leukemia (CLL), which identified the genomic deletion of miR-15a and miR-16-1 genes in CLL patients as an initiating event to promote CLL development [89, 90]. These studies have provided pioneering insight on the contribution of miRNA to cancer pathogenesis. Since then, mounting reports have provided evidence on the global miRNA differential expression signature in a multitude of human cancers, as well as elucidated the underlying molecular role of individual miRNA in tumorigenesis.

The widespread underexpression of miRNAs is a common phenomenon in cancers, albeit expressions of certain miRNAs being elevated in malignant cells [91]. Dysregulation of miRNAs in cancer can be due to multiple mechanisms, for instance, genomic lesions at miRNA loci, epigenetic regulation, transcriptional control by perturbed signaling pathway or defect in miRNA processing machinery. Regarding the function of miRNAs in cancer, it should be noted that one single miRNA can putatively target dozens or even hundreds of mRNAs to flexibly control

many biological processes. Also, miRNA expression is largely cellular context dependent, implying that one deregulated miRNA may display unequal role in different types of cancer. From initial target prediction by computational algorithms to meticulous experimental validation of interaction with target mRNA and effect on its expression, miRNAs that demonstrate specific roles in cancers are critically associated with their downstream targets. MiRNAs display oncogenic property when they negatively regulate tumor suppressors. On the other hand, miRNAs function as tumor suppressors when they target oncogenes. Some examples of oncogenic and tumor suppressor miRNAs are discussed below.

3.5.1 Oncogenic miRNAs

3.5.1.1 MiR-17-92

Mir-17-92, also known as oncomir-1, is a polycistronic miRNA cluster that contains 6 individual miRNAs, namely miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1. The genomic location of miR-17-92 is on chromosome 13q31 and its pri-miRNA transcript contains six stem-loop hairpin structures that are ultimately processed into the 6 mature miRNAs. Mir-17-92 cluster plays vital role in development of heart, lung and immune system [92]. The chr.13q31-q32 genomic region is often found to be amplified in hematopoietic malignancies and solid tumors [93], accounting for the aberrant up-regulation of miR-17-92 in these cancers. The oncogenic role of miR-17-92 was first described by He et al. in an in vivo mouse B-cell lymphoma model which showed that overexpression of miR-17-19b acted in concordance with c-Myc to accelerate lymphomagenesis. Specifically, miR-17-92 shortened the latency for tumor onset, repressed apoptosis and induced B-cell lymphomas that were mostly of progenitor B-cell origin, which was different from the mature B-cell containing c-Myc-derived lymphomas [94]. Later, O'Donnell et al. reported that transcription of the miR-17-92 cluster was directly transactivated by c-Myc [95], further strengthening the feedback regulation between oncogenic miRNA and signaling pathway to promote tumorigenesis. Subsequent studies continued to reveal the dynamic oncogenic role of miR-17-92 in different cancer context, including targeting the cyclin-dependent kinase inhibitor CDKN1A (p21) to sustain proliferation in gastric cancer [96], and repressing thrombospondin-1 (Tsp1) and connective tissue growth factor (CTGF) to stimulate angiogenesis in colon cancer [97].

3.5.1.2 MiR-21

The genomic location of miR-21 gene is on chromosome 17q23 and miR-21-5p is the abundant mature form of this miRNA. MiR-21 was first observed to be highly upregulated in human glioblastoma and depletion of miR-21 in cultured glioblastoma cells

activated caspase-dependent apoptosis, suggesting the antiapoptotic role of miR-21 in glioblastoma development [98]. Later studies further revealed the frequent upregulation of miR-21 in hematopoietic malignancies and other solid tumors [99]. No clear evidence has indicated that amplification of chr.17q region is closely associated with increased expression of miR-21 in cancers. On the contrary, transcriptional activation downstream of oncogenic signaling cascade and post-transcriptional control are implicated in elevating miR-21 expression. In myeloma cell line models, addition of IL-6 stimulated Stat3 activation which then bound to upstream enhancer region of miR-21 gene and induced its expression [100]. In MDA-MB-468 breast carcinoma cell line model, TGF-β and BMP accelerated the processing of pri-miR-21 into its mature form through the recruitment of SMAD and the Drosha microprocessor complex component RNA helicase p68, thereby elevating miR-21 expression in a post-transcriptional manner [101]. MiR-21 behaves as an universal oncomiR in human cancers; for example, it can target the well-known tumor suppressor PTEN in HCC and inhibition of miR-21 in cultured HCC cells increases PTEN expression with strong inhibitory function on cell proliferation, migration and invasion [102]. Other documented targets of miR-21 in human cancers include programmed cell death protein 4 (PDCD4) [103], tropomyosin 1 (TPM-1) [104], reversion-inducing cysteine-rich protein with kazal (RECK) [105] and Sprouty2 (SPRY2) [106].

3.5.1.3 MiR-10b

The genomic location of miR-10b gene is on chromosome 2q31 and miR-10b-5p is the abundant mature form of this miRNA. Upregulation of miR-10b was first discovered in metastatic breast cancer and later observed in other malignancies [107]. The pro-metastatic role of miR-10b is particularly compelling. In the initial study performed by Ma et al., they found that miR-10b was specifically upregulated in the human metastatic breast cancer cell line MDA-MB-231 as much as 50-fold than the non-metastatic breast cancer cell line MCF-7 [108]. Through a series of *in vitro* and *in vivo* functional studies, they confirmed the critical involvement of miR-10b in breast cancer migration, invasion and distant organ metastasis. More importantly, miR-10b was transcriptionally induced by the cancer invasiveness-related transcription factor TWIST1 through direct binding to the E-box sequences on miR-10b putative promoter. MiR-10b targets HOXD10 and leads to robust expression of RhoC to promote cell migration and invasion, suggesting that HOXD10/RhoC axis may be a critical downstream mediator of miR-10b's pro-metastatic role [108]. Interestingly, in glioblastoma, upregulation of miR-10b promotes cancer cell growth by reducing cell-cycle arrest and apoptosis. Gabriely et al. identified several cell-cycle regulators as direct targets of miR-10b, including Bim, AP-2γ, p21 and p16; and HOXD10 was not regulated in the context of glioblastoma [109]. The simultaneous regulation of these targets by miR-10b suggests that miR-10b may also function as a tumor promoting miRNA in addition to its pro-metastatic capability.

3.5.2 Tumor Suppressor miRNAs

3.5.2.1 MiR-200 Family

The miRNA-200 family comprises five miRNAs, namely miR-200a, miR-200b, miR-200c, miR-141 and miR-429. MiR-200b-200a-429 gene is clustered together and locates on chromosome 1p36, whereas miR-200c-141 gene locates on chromosome 12p13. The two clusters are expressed as polycistronic pri-miRNA transcripts, and their expressions may not be highly correlated. MiR-200 family members are frequently found to be down-regulated in many human cancers [110]. Functionally, miR-200 family members are well-known for their role in inhibiting epithelial-to-mesenchymal transition (EMT), which is a fundamental step of cancer metastasis. The EMT process is characterized by the loss of proteins required for maintaining epithelial phenotype, such as E-cadherin abundantly present between cell-cell adherens junctions. E-cadherin expression is in turn fine-tuned by epigenetics, and also its transcription repressor ZEB [111]. The observation that miR-200 family is implicated in maintaining epithelial phenotypes first arose from studies of somatic cell differentiation in mouse and zebrafish models, which clearly revealed the abundance of miR-200 family in skin epidermal cells and sensory epithelial structures [112]. In cancer cell models, miR-200b can target ZEB2 through multiple binding sites on its 3′UTR, and ectopic overexpression of miR-200c can target ZEB1 and restore E-cadherin expression [113, 114]. More importantly, Gregory et al. have shown that in human Madin Darby canine kidney (MDCK) epithelial cells, inhibiting miR-200 family members triggered EMT with acquisition of migratory property while enforced expression of miR-200 family members alone prevented TGF-β-induced EMT, both processes rely on ZEB1 and ZEB2 to mediate the phenotypic changes [114]. Interestingly, a feed-forward regulation between ZEB1 and miR-200c to stabilize EMT in cancer cells is suggested by Burk et al., who demonstrated that ZEB1 can suppress transcription of miR-200c-141 through binding to its promoter [115]. These findings shed light into miR-200 family-mediated anti-metastatic function through promoting EMT during malignant tumor progression.

3.5.2.2 Let-7 Family

The lethal-7 (let-7) miRNA family is large and comprises 12 family members, namely let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7g, let-7i and miR-98. The genomic locations of let-7 family members are on 8 different chromosomes. These 12 mature miRNAs share altogether 9 distinct let-7 seed sequences, suggesting that their sets of targets may partially overlap. Let-7 is first found to be down-regulated in human lung cancer cell lines and primary lung cancer tissues, and increasingly more studies identified the loss of expression of let-7 in other human cancers [116]. Several critical oncogenes are targets of let-7 miRNAs, implying an unequivocal tumor suppressive role of let-7. For example, K-RAS and N-RAS 3′UTR harbors multiple conserved let-7 binding sites and

reporter assays confirmed their putative interaction. Upon transfection of let-7a RNA mimics, RAS protein was dramatically reduced [117]. In primary lung cancer specimens, a reciprocal expression of RAS proteins and let-7 is observed, supporting the pathological relevance of let-7c in repressing RAS proteins [117]. Let-7 is also evidenced to target MYC [118], but MYC may also control the transcription of let-7 miRNAs through direct binding to promoters of let-7a-1/let-7f-1/let-7d cluster, thereby forming an autoregulatory loop in Myc-driven carcinogenic cascade [119]. Another let-7 targeted onco-protein is high mobility group A2 (HMGA2), which is a transcription factor associated with chromatin to modulate its architecture for transcription regulation. HGMA2 is frequently up-regulated in non-small-cell carcinoma of the lung and several benign mesenchymal tumors. Ectopic over-expression of HGMA2 can rescue the growth-suppressive effect mediated by let-7 in lung cancer cell models. Interestingly, HGMA2 gene is often characterized by having mutations that result in truncation of its 3′UTR. The truncated transcript lack let-7 binding sites and therefore enables it to escape repression by let-7 miRNAs [120]. Taken these studies together, they highlight the master tumor suppressive role of let-7 miRNAs through repressing multiple onco-proteins.

3.5.2.3 MiR-125b

The genomic location of miR-125b gene is on chromosome 11q24 and miR-125b-5p is the abundant mature form of this miRNA. Expressions of miR-125b were down-regulated in many human cancers including liver cancer [121], melanoma [122] and breast cancer [123]. In primary HCCs, miR-125b expression level is threefold lower than that of the nontumorous liver counterparts; and 70 % of HCC cases examined showed down-regulation of miR-125b. Introduction of miR-125b into HCC cells dramatically inhibited their proliferation and migratory ability. LIN28B, which is a miRNA-binding protein, is further identified as target of miR-125b based on *in silico* prediction and miRNA luciferase reporter assay [121]. Later study by Gong et al. revealed additional targets of miR-125b in liver cancer, including anti-apoptotic proteins MCL-1, BCL and interleukin (IL)-6R which play causative role in mitochondrial apoptotic pathway [124]. Interestingly, miR-125b has recently been described as an "epi-miRNA" through negatively regulating the H3K9 methyltransferase SUV39H1 in HCC. Fan et al. showed that upon overexpression of miR-125b in HCC cell lines, SUV39H1 protein level was reduced. More importantly, mRNA expression levels of SUV39H1 and miR-125b were negatively correlated in clinical HCC tissues, implying SUV39H1 overabundance may be due to miR-125b deregulation [125].

3.5.2.4 MiR-139

The genomic location of miR-139 gene is on chromosome 11q13 and miR-139-5p is the abundant mature form of this miRNA. Down-regulation of miR-139 has been reported in HCC [126] and gastric cancer [127]. Mir-139 was first described as an

anti-metastasis miRNA through its negative regulation on the Rho kinase ROCK2 in HCC. Wong et al. have found that miR-139 expression is reduced by more than tenfold in primary HCCs as compared with their nontumorous liver tissues; and down-regulation of miR-139 is noted in up to 76 % of human HCCs. Strikingly, further down-regulation of miR-139 is observed in the venous metastases and distant extrahepatic HCC metastasis as compared with the corresponding primary tumors. Down-regulation of miR-139 is significantly associated with aggressive tumor behavior and poor prognosis of HCC patients [126]. Consistent to the close implication of miR-139 in HCC metastasis, ROCK2, whose overexpression modulates cytoskeletal reorganization to promote cell migration, is shown to be negatively regulated by miR-139 [126]. The miR-139/ROCK2 regulatory axis represents an important mechanism to understand how miRNA deregulation contributes to cancer metastasis. In another study performed by Shen et al., they demonstrated that in colorectal cancer, increased expression of miR-139 can target type I insulin-like growth factor receptor (IGF-IR) which consequently alters the MEK/ERK/NF-κB/signaling and attenuates matrix metalloproteinase 2 (MMP2) transcription. Degradation of extracellular matrix by MMPs enables cancer cells to invade and disseminate from their primary site [128]. The identified miR-139/IGF-IR/MMP2 axis further enriches our understanding of the anti-invasion role of miR-139 during malignancy development.

3.6 Reciprocal Regulation of EZH2 and miRNA in Cancers

In the previous sections, we have reviewed the crucial involvement of EZH2 and miRNAs in the pathogenesis of cancer. EZH2 and miRNAs are dependent on each other since they are capable of reciprocally regulating themselves, further providing fine adjustment of their own expressions to achieve cancer-specific roles. Negative regulation of EZH2 by miRNAs is plausible and identified as an underlying cause of its up-regulation during cancer progression. EZH2 simultaneously exerts epigenetic control over a wide-range of miRNAs to promote cancer proliferation and metastasis. Remarkably, EZH2 can orchestrate concordant expressions of PcG-targeting miRNAs to self-reinforce the PcG epigenetic silencing machinery, suggesting an intricate regulatory loop between miRNA and EZH2 is of utter importance for cancer development (Fig. 3.5). In this section, we will discuss examples of miRNAs targeting EZH2, as well as how EZH2 epigenetically modulates expressions of a subset of miRNAs to promote cancer development.

3.6.1 MiR-101 Negatively Regulates EZH2

Study by Varambally et al. is the first to report that miR-101 can target EZH2 in breast and prostate cancer models [129]. MiR-101 negatively regulates EZH2 through binding to EZH2 3′UTR on two putative sites. Ectopic overexpression of

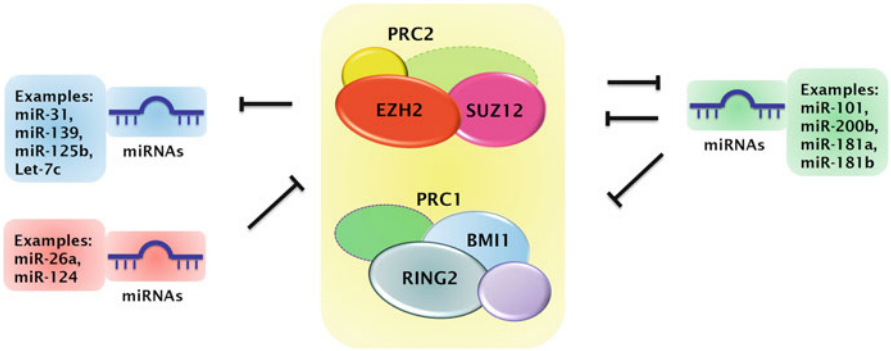

Fig. 3.5 EZH2 and miRNAs reciprocally regulate each other in cancer pathogenesis. EZH2 and miRNA are closely connected in part due to their ability of regulating each other to achieve cancer-specific expressions. Upregulation of EZH2 is attributed to loss of expressions of miR-101, miR-26a and miR-124 in different malignancies. Conversely, EZH2 also epigenetically represses tumor and metastasis suppressive miRNAs to facilitate cancer growth and progression. More remarkably, EZH2 and miRNAs (e.g. miR-101, miR-200b, miR-181a, miR-181b) form feed-forward regulatory loop to reduce expression of PRC-targeting miRNAs while reinforcing PcG-mediated epigenetic activity in cancers

miR-101 in prostate cancer cell line DU145 and breast cancer cell line SKBr3 not only reduced EZH2 and other PRC2 proteins expression level, but also profoundly inhibited cell proliferation, anchorage-independent growth and invasion [129]. Down-regulation of miR-101 is frequent in human cancers due to genomic loss of miR-101-1 and miR-101-2 loci on chromosome 1 and chromosome 9, respectively. More importantly, miR-101 expression was inversely correlated with EZH2 expression in clinical metastatic prostate cancer specimens, suggesting that the loss of miR-101 and concomitant upregulation of EZH2 play an important role in cancer aggressiveness [129].

3.6.2 MYC-Repressed miR-26a Negatively Regulates EZH2

During myogenesis, miR-26a is elevated to repress EZH2, thereby stimulates skeletal muscle cell differentiation [130]. Interestingly, a similar regulation of EZH2 by miR-26a also exists in lymphoma to promote lymphomagenesis. Sander et al. used a murine MYC-driven lymphoma model and identified a subset of MYC-repressed miRNAs. Upon further validation, they confirmed that miR-26a exhibited the most inhibitory effect on proliferation when it was overexpressed in the human Burkitt lymphoma cell lines Namalwa and Raji. From *in silico* prediction and gene expression profiling of miR-26a overexpressing cell lines, they validated EZH2 as a direct target of miR-26a through binding to EZH2 3′UTR on one putative site [131].

Further studies also revealed that miR-26a expression was negatively correlated with EZH2 expression in other cancers [132, 133], indicating that miR-26a regulates EZH2 in common tumorigenesis.

3.6.3 MiR-124 Negatively Regulates EZH2

A recent study by Zheng et al. demonstrated that miR-124 can target both EZH2 and the Rho kinase ROCK2 to drive HCC metastasis [134]. The expression level of miR-124 is reduced in most of the HCC cell lines examined, being the lowest in the metastatic HCC cell line MHCC-LM9. Downregulation of miR-124 is observed in 67 % primary HCCs as compared with their adjacent non-tumorous tissues. Interestingly, miR-124 directly regulates both EZH2 and ROCK2 through binding to a single putative site on their 3′UTR. Phenotypically, overexpression of miR-124 dramatically inhibits HCC cell migration and invasion by perturbing actin cytoskeleton reorganization in a manner analogous to suppression of EZH2 or ROCK2 [134]. Downregulation of miR-124 can be due to DNA promoter methylation [135], or loss of transcription induction by hepatocyte nuclear factor 4α (HNF4α) [136].

3.6.4 EZH2 Suppresses Anti-metastatic miRNAs in Cancers

EZH2 is known to epigenetically repress multiple anti-metastatic protein-coding genes to drive cancer progression [5, 67]. Remarkably, EZH2 can also epigenetically silence miRNAs with tumor and metastasis suppressive functions. In our previous study, we have demonstrated that stable knockdown of EZH2 in HCC cell lines significantly induces expression of a plethora of miRNAs. A specific subset of miRNAs is commonly re-expressed in three HCC cell lines examined, suggesting that their silencing generally involved EZH2. These miRNAs include the anti-metastatic and tumor suppressor miR-139-5p, miR-125b, miR-101, miR-511, miR-99a*, let-7c, and miR-200b, all of which are significantly downregulated in human HCCs [31]. *In silico* miRNA target prediction and pathway enrichment analysis have unraveled the combinational effect exerted by the EZH2-miRNA axis on key cell motility-associated pathways, including focal adhesion and adherens junction that are crucial for cancer cell invasion and metastasis. Many components of the RhoGTPase-associated cytoskeleton reorganization axis are predicted targets of EZH2-regulated miRNAs, and ROCK2 and RhoA expressions are significantly altered together with inhibition of stress fiber formation upon EZH2 knockdown in HCC cells [31]. Taken together, this study has provided evidence on a predominant and global role of EZH2-miRNA axis in driving liver cancer metastasis.

3.6.5 PcG Suppresses miR-31 to Activate NF-κB Signaling in Cancers

In tumor cells, constitutive activation of NF-κB signaling and deregulation of its target genes (e.g. proinflammatory cytokines and chemokines) play a pivotal role to promote carcinogenesis. Yamagishi et al. revealed that aberrant activation of NF-κB signaling in adult T cell leukemia (ATL) is partly due to PcG-mediated silencing of miR-31 [137]. MiR-31 is downregulated in ALT samples compared to control CD4+ T cells from healthy donors. Computational algorithms and luciferase reporter assay validated NF-κB-inducing kinase (NIK), which is known to phosphorylate IKKα and thus noncanonically activates NF-κB signaling, as a direct target regulated by miR-31 through binding to two putative sites on NIK's 3′UTR. The loss of miR-31 in a small proportion (12.5 %) of ATL patients could be attributed to genomic deletion of miR-31 gene on chromosome 9p21.3. More intriguingly, miR-31 is under YY1/PcG-mediated epigenetic regulation. Promoter region of miR-31 harbors clusters of YY1 binding motifs and, upon knockdown of YY1, miR-31 expression is induced with concomitant decreased occupancy of YY1 and derecruitment of EZH2 at the promoter region. Consistently, knockdown of PRC2 components restores miR-31 transcription and increases intracellular levels of NIK. In breast cancer, miR-31 acts as metastasis suppressor [138], but its impact on cell movement is not seen in ALT. This study highlights the significant role of PRC2-miR-31-directed activation of NF-κB signaling for ATL development, and emphasizes the capability of miRNAs to exert cell-type and tissue-specific effect.

3.6.6 EZH2 Suppresses PRC1-Targeting miRNAs in Cancers

In human cancers, both PRC2 and PRC1 proteins are often upregulated. Cao et al. undertook the study to explore whether miRNAs mediate the synergy of PcG proteins [139]. To identify miRNAs regulated by EZH2, they transiently knockdown EZH2 in the prostate cancer cell line DU145 and profiled the global miRNA expression changes with other four benign epithelial cell lines. Inhibition of EZH2 reexpressed many miRNAs and they further concentrated on a subset of miRNAs that might, by *in silico* prediction, target PRC1 proteins BMI1 and SUZ12. Among the 14 miRNAs identified, they validated that miR-181a, miR-181b, miR-200b and miR-200c could directly target RING2; and miR-203, miR-200b and miR-200c could directly target BMI1. In clinical advanced stage prostate cancer tissues, the expressions of these miRNAs were anti-correlated with EZH2, BMI1, RING2 and the PRC1-specific histone modification H2A ubiquitylation [139], indicating that coordination of PRC1 proteins by EZH2-regulated miRNAs play an important role in prostate cancer progression.

As discussed previously, miR-200 family downregulation by DNA methylation promotes EMT for cancer cell migration and invasion [114]. Cao et al. have revealed

that miR-200b and miR-200c targeting PRC1 proteins are silenced by EZH2 in cancer [139]. Au et al. have also identified miR-200b as one of EZH2-regulated miRNA targets in liver cancer [31]. Interestingly, Iliopoulos et al. have indeed demonstrated that miR-200b is a critical regulator of breast cancer stem cell growth and function through inhibiting the PRC2 protein SUZ12, and to a lesser extent BMI1 of PRC1 [140]. In accord with the ability of a single miRNA pleiotropically regulates multiple targets, miR-200 family may serve as an important epigenetic and metastasis regulator simultaneously during tumorigenesis. Taken these studies together, they have enriched current knowledge on the regulation of PRC proteins by miRNAs in the cancer specific context and more importantly, they implicate a PcG-miRNA self-regulatory loop to maintain an epigenetic program essential to cancer development.

3.7 Concluding Remarks

Given the significant impact of upregulated PcG activity and aberrant miRNA expressions in cancer pathogenesis, it is highly relevant to investigate them and ideally, the new knowledge can help devise new clinical biomarkers for cancer detection or more effective therapeutic approaches for cancer treatment. Exploiting the small-molecule inhibitor 3-deazaneplanocin A (DZNep) targeting EZH2 appears to achieve antitumor effect in certain cancer models *in vitro* and *in vivo* [141, 142]. Systemic administration of miRNA mimetics [143], antagomirs [144], or delivery of tumor-suppressing miRNA based on adeno-associated virus (AAV) [145] provide proof-of-concept support for manipulating miRNA as a powerful and nontoxic anticancer strategy. Challenges ahead remain to completely translate these experimental findings into clinical implementation. It is anticipated that future directions for anticancer strategies will incorporate both epigenetic therapy and miRNA targeting.

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Chapter 4
Epigenetics and MicroRNAs in Renal Cancer

Shahana Majid, Sharanjot Saini, Guoren Deng, and Rajvir Dahiya

Abstract Epigenetic states are orchestrated by several converging and reinforcing signals, including DNA methylation, histone modifications and non-coding RNAs. Growing evidence indicates that acquired epigenetic abnormalities participate with genetic alterations to cause cancer. In this review we describe recent advances in the field of cancer epigenomics and microRNAs (miRNAs) with special emphasis on renal cancer. We discuss whether epigenetic changes are the cause or consequence of cancer initiation and the use of epigenetic biomarkers and miRNAs for cancer diagnosis or prognosis. Finally we address the potential of epigenetic based anti-cancer therapeutic strategies.

Keywords Epigenetics • MicroRNA • Renal cancer

4.1 Background

The term 'epigentics' was originally coined by Conrad Waddington in 1942 for the molecular mechanisms that convert genetic information into observable traits or phenotypes during development [1]. By contrast, Arthur Riggs et al. defined epigenetics as "the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" [2]. The term may be currently defined as the mechanisms that initiate and maintain heritable patterns of gene function and regulation without affecting the sequence of the genome [3]. The sum total of all epigenetic information is termed the 'epigenome' and comprises

S. Majid • S. Saini • G. Deng • R. Dahiya, Ph.D. (✉)
Department of Urology, Urology Research Center (112F), V A Medical Center and UCSF, 4150 Clement Street, San Francisco, CA 94121, USA
e-mail: rdahiya@urology.ucsf.edu

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some of the instructions directing the genome to express genes at particular places and times [4, 5]. Unlike the genome, the epigenome is highly variable between cells and fluctuates in time according to conditions even within a single cell. Each of us has essentially one genome, however each cell type in each individual is believed to have a distinct epigenome that reflects its developmental state [6]. The epigenetic state of a cell is affected by developmental as well as environmental influences that may leave epigenetic traces which the cell remembers, referred to as cellular memory [7]. Thus the epigenome provides a crucial interface between the environment and the genome. Recent breakthroughs in the understanding of epigenetic mechanisms provide evidence that they are fundamental to the regulation of many cellular processes, including gene and microRNA expression, DNA-protein interactions, suppression of transposable element mobility, cellular differentiation, embroygenesis, X-chromosome inactivation and genomic imprinting [8]. The disruption of epigenetic changes underlies a wide variety of pathologies including cancer [9]. The cancer epigenome is characterized by global changes in DNA methylation including hypomethylation, promoter specific hypermethylation, histone modification, chromatin-modifying enzyme expression profiles and global dysregulation of non-coding microRNAs (miRNAs). These aberrations confer a selective growth advantage to neoplastic cells, apoptotic deficiency and uncontrolled cell proliferation, leading to cancer initiation and progression. For didactic purposes, epigenetic mechanisms may be grouped into DNA methylation, histone modification and remodeling and miRNAs. In this review, we will describe these mechanisms with an emphasis on alterations of the epigenome taking place in renal cancer.

4.2 DNA Methylation

Aberrant DNA methylation is the best characterized cancer-related epigenetic modification. DNA methylation occurs predominantly at the symmetrical dinucleotide CpG sites [10] that are scattered throughout the genome at a lower-than-expected frequency. However, in certain areas of the genome, a high concentration of CpG dinucleotides is found, and are referred to as “CpG islands” (CGIs) [11]. In a normal differentiated cell, CpG loci disseminated across the genome are highly methylated, whereas most promoter CGIs are protected from methylation inside their boundaries [11]. In general CGI methylation is associated with gene silencing. Gene silencing associated with CGI promoter methylation may be due to restricted access of transcription factors or binding of methylcytosine-binding proteins (MBD), which cooperate with DNMTs and histone deacetylases (HDACs) [12]. An important role in the regulation of gene expression has also been credited to low density CpG regions located in the vicinity of CpG islands, the so-called “CpG island shores” [13, 14]. These are sequences up to 2 kb distant from CpG islands, that are associated with gene expression. Remarkably, methylation patterns at CpG island shores are mostly tissue-specific and cancer-associated alterations in these patterns occur at sites that vary normally in tissue differentiation [14]. Differentially

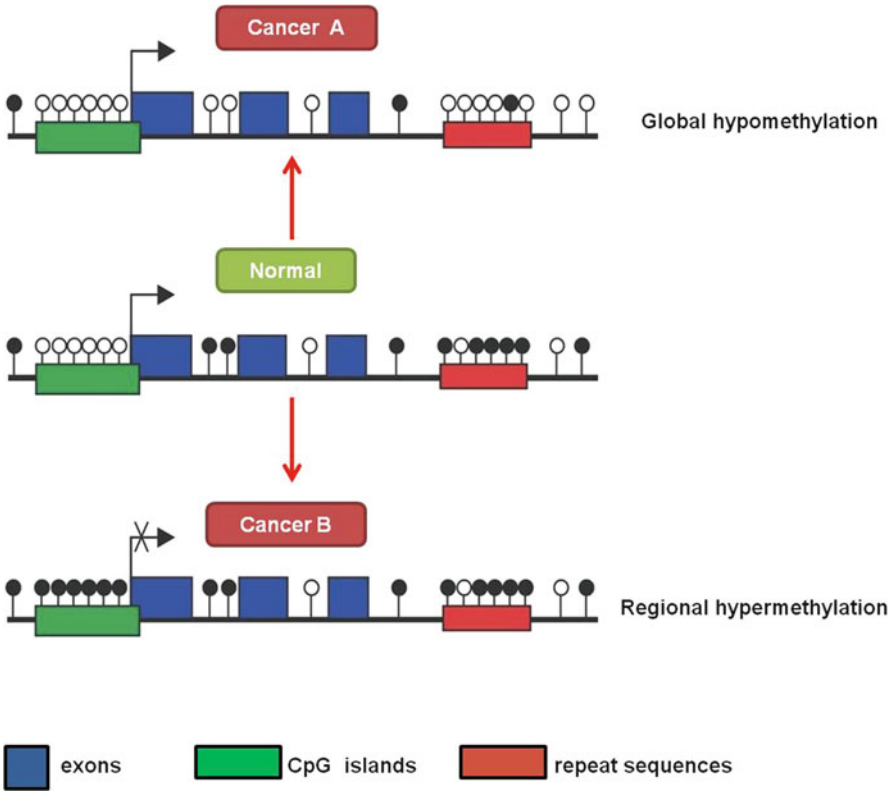

Fig. 4.1 Two epigenetic pathways of transformation from normal cells to cancer cells. In normal cells, DNA is unmethylated in CpG islands, while in repeat sequences and CpG sparse regions, DNA is heavily methylated. When DNA is less methylated in repeat sequences and CpG sparse regions, cells are transformed (Cancer A with global hypomethylation). If DNA is heavily methylated in CpG islands, cells are also transformed (Cancer B with regional hypermethylation)

methylated CpG island shores are sufficient to distinguish between specific tissues and are conserved between human and mouse [13, 15]. Aberrations in DNA methylation include both global and gene-specific hypomethylation as well as gene-specific CpG island promoter hypermethylation [3, 16] (Fig. 4.1). Since global DNA hypomethylation and promoter-specific hypermethylation can be commonly observed in benign neoplasias and early-stage tumors, it is becoming apparent that epigenetic deregulation may precede the classical preliminary transforming events such as mutations in tumor suppressors, protooncogenes and genomic instability [17]. These aberrations have also have been considered to be the earliest events in the process of tumorigenesis [18]. The impact of gene-specific alterations in DNA methylation depends on the function of the affected gene and the type of alteration. Whereas promoter hypomethylation may cause activation of proto-oncogenes, hypermethylation induces silencing of cancer-related genes with tumor suppressive properties [18]. On the other hand, genome-wide hypomethylation may lead to

Table 4.1 Genes and microRNAs methylated in renal cell carcinoma

Gene	References
WNT7a	[27]
TCF21	[28]
SLC34A2	[29]
OVOL1	[29]
DLEC1	[29]
TMPRSS2	[29]
SST	[29]
BMP4	[29]
GATA5	[30]
Rap1GAP	[31]
KLHL35	[32]
QPCT	[32]
SCUBE3	[32]
ZSCAN18	[32]
CCDC8	[32]
FBN2	[32]
ATP5G2	[32]
PCDH8	[32]
CORO6	[32]
DLEC1	[33]
miR-34a	[34]
miR-34b/c	[34]
miR-9	[35][a]

[a]For other methylated genes in RCC, please see Ref. [36]

genomic instability in repetitive sequences, especially at pericentromeric regions, predisposing to abnormal recombination, facilitating translocations, deletions, and chromosomal rearrangements [19–21].

Renal cell carcinoma (RCC) is genetically and histopathologically a heterogeneous disorder. The most common subtype of RCC is clear cell RCC (ccRCC; approximately 75 %) and the next most frequent subtype is papillary RCC (pRCC; approximately 15 %) [22]. The most frequent genetic abnormality in ccRCC is inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene [23] and promoter methylation of tumor suppressor geness (TSGs) is common in both subtypes of RCC. The *VHL* and $p16^{INK4a}$ TSGs are inactivated by promoter hypermethylation in up to 20 % of clear cell [24] and 10 % of all RCC [25]. The *RASSF1A* and the *Timp-3* genes are hypermethylated in 27–56 % [26] and 58–78 %, of primary RCCs respectively [26]. Table 4.1 provides an overview of the commonly methylated genes in renal cancer based on the published reports. A survey of published work in 2010 by Morris and Maher [36] has identified 58 genes that are methylated in RCC and 43 of these genes had a mean combined methylation/mutation rate of over 20 % (Ref. [36]). Cancer genome projects such as TCGA (http://cancergenome.nih.gov/l) and CAGEKID (http://www.icgc.org/icgc/cgp/65/812/817) have elected to define the mutational status and methylation profile of RCC. Hence large amount of data will

be available to identify frequently methylated genes in RCC in the near future. Our group has also reported that various genes such as DNA mismatch repair genes [37], E-cadherin [38], gamma-catenin [39] and BTG3 [40] are silenced through promoter hypermethylation in renal cancer. We have also published extensively on the promoter methylation status of genes involved in the Wnt signaling pathway in renal cancer. Oncogenic activation of the Wnt pathway drives expression of genes that contribute to proliferation, survival and invasion. Inhibitors of this pathway can be divided into two functional classes, sFRP proteins that bind directly to Wnt and prevent its binding to frizzled receptor and the Dickkopt (DKK) proteins which bind to LRP component of the Wnt receptor complex. The *sFRP-1, sFRP-2, sFRP-4, sFRP-5* and related Wif genes are all frequently methylated in RCC [41–44], as are the DKK genes [45, 46]. An interesting finding by our group is that *sFRP1* is unmethylated/hypomethylated and thus over-expressed in metastatic renal tumors [47] compared to primary tumors where in its expression is attenuated by promoter hypermethylation [41]. Another study from our group by Yamamura et al. [48] challenged the Wnt inhibitory role of *sFRP2* and reported that overexpression of *sFRP2* activates the canonical Wnt pathway, promoting cell growth through diverse signaling cascades in renal cancer cells [48].

4.3 Chromatin Remodeling and Histone Modifications

The coiling of DNA around nucleosome particles is the basis for organization of eukaryotic genomes. Each nucleosome encompasses ~147 bp of DNA wrapped around an octamer of histone proteins. The core histones H2A, H2B, H3 and H4 bind together (two H2A-H2B dimers and one H3-H4 tetramer) to form the nucleosome. The core histones are small basic proteins containing a globular domain and a flexible charged NH2 terminus known as the histone tail [49]. Regulation of gene expression occurs through posttranslational covalent modifications of the histone tails including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP ribosylation [49, 50]. Generally certain histone modifications such as acetylation or phosphorylation are thought to change chromatin structure by altering the net positive charge of the histone proteins, thereby making the underlying DNA sequence accessible [51]. Alternatively, histone modifications can be recognized by specific protein domains (e.g., bromodomains, Tudor domains, chromodomains), which in turn might enforce or stabilize the recruitment of additional factors [52, 53]. Posttranslational modifications to histone tails govern the structural status of chromatin and the resulting transcriptional status of genes within a particular locus. These modifications are reversible and controlled by a group of enzymes including histone acetyltransferases (HATs) and deacetylases (HDACs), methyltransferases (HMTs), demethylases (HDMs), kinases, phosphatases, ubiquitin ligases and deubiquitinases, SUMO ligases and proteases which add and remove these modifications [8, 49]. In relation to transcriptional state, the human genome can be roughly divided into two distinct chromatin conformation states: euchromatin,

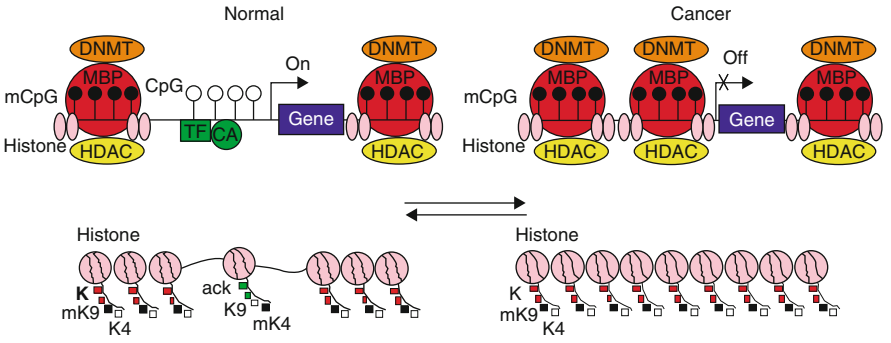

Fig. 4.2 DNA methylation status in the promoter and the related chromatin structure. In normal cells, the CpG sites adjacent to transcription start site are unmethylated. The transcriptional machinery is activated by the binding of transcriptional factor (TFs) and co-acting factors (CAs) in this region. The gene promoter shown on the *upper left* is transcriptionally active. In upstream and downstream regions, DNA is methylated by DNA methyltransferases (DNMTs). In these regions, methylcytosine-binding proteins (MBPs) that bind to methylated CpG sites recruit histone deacetylases (HDACs) and histone methyltransferases to form a complex. *Left bottom* shows the related chromatin structure around the transcriptionally active, unmethylated promoter. The lysine residues in the tails of histone H3 are acetylated (acK). Lysine 4 is methylated (mK4) and lysine 9 is unmethylated (K9). These changes contribute to open and relaxed conformation of the chromatin allowing key components of the transcription apparatus accessible to the promoter. In the upstream and downstream regions, the lysine residues are deacetylated (K), demethylated (K4) and methylated (mK9) respectively and the chromatin structure have closed and dense conformation. In cancer cells, shown in the *upper right*, DNA methylation spreads toward the promoter regions near the transcriptional start site, resulting in transcriptional silencing. These events result in closed and dense chromatin conformation making it difficult for the key components of gene transcription apparatus to bind to the promoter

which has an open structure and is transcriptionally active and heterochromatin, which is densely compacted and transcriptionally inert [54]. Euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79. In contrast heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation [8, 54]. The notion of heterochromatin as transcriptionally inactive has been challenged by the discovery of numerous noncoding RNAs (ncRNAs) derived from heterochromatic loci [55]. Well-known examples of this phenomenon in humans are the ncRNAs *XIST* and *HOTAIR* [56, 57]. Histone modifications are predictive for gene expression as actively transcribed genes are characterized by enriched levels of H3K4me3, H3K27ac, H2BK5-azacytidine (H2BK5ac) and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene [58]. Therefore histone modifications influence chromatin structure which plays an important role in gene regulation and carcinogenesis (Fig. 4.2).

Genome-wide studies have revealed that various combinations of histone modifications in a specific genomic region can lead to a more ‘open’ or ‘closed’ chromatin structure resulting in the activation or repression of gene expression. Disruption of normal patterns of histone modifications is a hallmark of cancer [12, 59]. One of the most characteristic examples is the global reduction of H4K20 trimethylation (H4K20me3) and H4K16 acetylation (H4K16Ac), along with DNA hypomethylation,

at repeat sequences in many primary tumors [12]. Furthermore, genes encoding for histone-modifying enzymes have been also reported to be mutated in ccRCC [60]. Mutated genes have been implicated in chromatin regulation through nucleosome repositioning and histone tail modification. *PBRM1*, which was found to be mutated in nearly 40 % of human RCCs [61, 62], is a component of the Polybromo BRG1-associated factor complex (PBAF, SWI/SNF-B). PBAF, like SWI/SNF, functions as a nucleosome remodeler and was shown to be involved in transcriptional regulation (24–26). Less common mutations were also identified in two methyltransferases, SETD2 and MLL2, and two demethylases, UTX (KDM6A) and JARID1C [KDM5C Ref. [60]]. Deletion of chromosome 3p is a common finding in ccRCC associated with the loss of VHL at 3p25 and can also affect SETD2 and PBRM1, which are located at 3p21 [63]. SETD2 mediates the trimethylation of H3K36 [64], a histone mark that is placed during transcription and may be important for maintaining faithful transcription [65], whereas MLL2 mediates H3K4me3, a mark associated with active transcription. UTX demethylates H3K27me3 [66, 67], a histone mark associated with repressed chromatin. Of interest, UTX associates with MLL2 [68], suggesting that demethylation of repressive modification is associated with transcriptional activation. The hypoxia response pathway has been shown to have a direct effect on histone modification. HIF upregulation is a feature of ccRCC and it was shown to activate several chromatin demethylases, including JMJD1A (KDM3A), JMJD2B (KDM4B), JMJD2C (KDM4C), and JARID1B (KDM5B), all of which are directly targeted by HIF [69–71]. Indeed, both JMJD1A and JMJD2B were found to be elevated in a RCC cell line with loss of VHL function [70], and the expression of JMJD1A was reported to be higher in RCC cancer tissue surrounding blood vessels, suggesting that JMJD1A is involved in tumor angiogenesis [72]. Reexpression of VHL in VHL-deficient cell lines increased H3K4me3 levels associated with decreasing levels of JARID1C, a target of HIF2a [62]. Silencing of JARID1C in VHL-deficient tumor cells augmented tumor growth in a xenograft mouse model, suggesting that JARID1C acts as a tumor suppressor. In contrast, hypoxia may increase methylation through HIF-independent mechanisms. Like HIF prolyl hydroxylase (PHD, EGLN3), histone demethylases are members of the dioxygenase superfamily, which requires oxygen as well as iron and 2-oxoglutarate for activity [73, 74]. In a manner analogous to stabilization of HIF via decreased hydroxylation, hypoxia was shown to suppress JARID1A (KDM5A) activity, resulting in increased H3K4me3 levels [75]. This suggests the hypothesis that loss of demethylases (and, by analogy, increased histone methylation) is part of a hypoxia phenotype that is selected for in RCC. This hypoxia phenotype, which is mimicked by *VHL* loss, would also be mimicked by loss of histone demethylase activity, which is a high-frequency event in RCC. Chromatin organization also influences HIF function. Studies of HIF induced under conditions of hypoxia showed preferential targeting of HIF to previously nucleosome depleted chromatin regions [76]. Moreover, the coexpression of SWI/SNF components BRG1, BAF170, and BAF57 augmented HIF activity from an HIF responsive reporter [77]. The extent to which mutations of epigenetic regulators influence chromatin or HIF targeting remains unknown.

4.4 MicroRNAs

MicroRNAs are small, non-protein-coding sequences thought to regulate >90 % of human genes by targeted repression of gene transcription and translation [78]. These endogenous, silencing RNAs have been shown to play important roles in development and differentiation [79, 80], cellular stress responses [81], and growing evidence has strongly implicated the involvement of miRNAs in carcinogenesis [82–84]. Specific subsets of miRNAs have also been shown to be dysregulated in various solid tumors [85, 86]. Due to their tremendous regulatory potential and tissue-specific and disease-specific expression patterns [87, 88], there is increasing evidence that miRNA expression profiles could be indicative of disease risk.

DNA hypermethylation of CpG sites within CpG islands is known to lead to the inactivation of many tumor-suppressive miRNAs [89–91]. One of the most common causes of tumor-suppressor miRNA loss is silencing of their primary transcripts by CpG-island hypermethylation [92–96]. The DNA methylation profile of tumors is useful to define tumor type, clinical prognosis and treatment response [19, 20]. Epigenetic silencing of miRNAs is also involved in the acquisition of an invasive phenotype and the development of metastasis [93]. Dysregulation of miRNA expression seems to be pivotal for RCC development and progression. Table 4.2 presents a list of miRNAs that are dysregulated in RCC. Depletion of tumor suppressor genes or upregulation of oncogenes has also been correlated with dysregulated expression of miRNAs in RCC. Our group has reported that several tumor suppressor miRNAs such as miR-1826 [102], miR-708 [100], miR-205 [104], miR-584 [105] are attenuated in RCC, where as oncogenic miR-21 was overexpressed [108]. There are controversial reports about the status of miRNA-34a in RCC. One study reported that inactivation of miR-34a correlates with its methylation status as they found methylation frequency of 58 % in RCC [34]. "Whereas in contrast, Liu et al. [109] reported increased levels of mir-34a caused loss of function of tumor suppressor *SFRP1* [which again is a controversial tumor suppressor in RCC [47]] indicating its oncogenic potential" [109]. However no functional analysis was

Table 4.2 MicroRNAs and their targets in renal cell carcinoma

microRNAs	Function	Target gene	References
miR-99a	Tumor suppressor	mTOR	[97]
miR-138	Tumor suppressor	Vimentin	[98]
miR-204	Tumor suppressor	MAP1LC3B	[99]
miR-708	Tumor suppressor	Survivin	[100]
miR-1	Tumor suppressor	transgelin-2	[101]
miR-133a	Tumor suppressor	transgelin-2	[101]
miR-1826	Tumor suppressor	CTNNB1, MAP2K1	[102]
miR-34a	Tumor suppressor	c-Myc	[103]
miR-205	Tumor suppressor	Src kinase	[104]
miR-584	Tumor suppressor	ROCK1	[105]
miR-23b	Oncogenic	Proline oxidase	[106]
miR-21	Oncogenic	PTEN	[107]

performed in either study. A recent study by our group investigated the functional effects of miR-34a in RCC [103]. It reported that overexpression of miR-34a inhibited cell invasion and suppressed the assembly and function of the c-Myc complex that activates or elongates transcription, indicating a tumor suppressor role in RCC [103]. Matching patterns between deregulated miRNAs and chromosomal aberrations have been reported in ccRCC [110]. On the other hand, miRNA deregulation might serve as an alternative mechanism for gene expression alterations due to chromosomal aberrations. This is well illustrated by the miR-204/211 family. Gain of chromosome 3q is a common finding in papillaryRCC that leads to upregulation of several genes including *C3orf58, CCDC50, DTX3L, PLD1, TRIM59, ECT2, RAP2B,* and *SERP1* that are targeted by miR-204/211 [Ref. [109]], whereas in ccRCC, miR-204/211 downregulation might be the mechanism causing upregulation of the same set of genes, since 3q gain is rare in ccRCC [109].

4.5 Interplay Between Epigenetic Factors

There is interplay between histone modifications and DNA methylation and the best example is the relationship between DNMT3L and H3K4. DNMT3L specifically interacts with histone H3 tails, inducing *de novo* DNA methylation by recruitment of DNMT3A, however this interaction is strongly inhibited by H3K4me. Furthermore, several histone methyltransferases have also been reported to direct DNA methylation to specific genomic targets by recruiting DNMTs [111, 112], helping in this way to set the silenced state established by the repressive histone marks. Moreover, histone methyltransferases and demethylases can also modulate the stability of DNMT proteins, thereby regulating DNA methylation levels [113, 114]. On the other hand, DNA methylation can also direct histone modifications. For instance, methylated DNA mediates H3K9me through MeCP2 recruitment [115]. MicroRNAs are also known to target the components of epigenetic machinery such as DNMTs, HDACs and polycomb genes [116]. Whereas, miRNAs may be affected by epigenetic changes, such as methylation of the CGIs and accompanying changes in histone modifications. miR-127 has been found to be attenuated in cancer cells by promoter hypermethylation and by a decrease in acetyl-H3 and methyl-H3K4 [95]. Genome-wide analysis of different cancer types has shown that global expression of miRNAs is influenced by DNA methylation and histone modifications [117].

4.6 Epigenetic and miRNA Biomarkers

Methylated DNA sequences provide attractive options for biomarkers for cancer detection and prognosis including RCC [118]. The last decade has provided an extensive map of the aberrant DNA methylation events occurring in cancer cells, particularly for the hypermethylated CpG islands of tumor suppressor genes (TSG) [19].

Consequently a myriad of DNA methylation-based biomarkers of many types of human neoplasias have been reported. Different RCC subtypes seem to display different gene sets deregulated by promoter hypermethylation [26], and a gene panel (*CDH1*, *PTGS2*, and *RASSF2*) identifying most frequent RCC subtypes in tissue samples has been evaluated [119]. The epigenomic data have helped highlight the unique profile of aberrant DNA methylation that defines each tumor type [120]. Epigenetic biomarkers are of particular interest as non-invasive biomarkers since methylated DNA can be detected from tumor cells sloughed into urine or blood. This has been shown with a three-gene panel (*APC*, *RARβ2*, *RASSF1A*) which detected RCC with high specificity and sensitivity [41, 121]. Moreover, *RASSF1A* promoter methylation might also prove useful for tumor surveillance/monitoring of RCC cancer patients [122]. Methylation of the Wnt pathway genes *SFRP1, SFRP2, SFRP4, SFRP5, DKK3* and *WIF1* have been detected in the serum of patients with corresponding tumor methylation and the frequency of methylation in serum correlated with increased grade and stage [41]. Therefore the detection of RCC-associated TSG methylation by analysis of serum or urine samples could have potential for early detection of RCC and for distinguishing benign and malignant renal cancers. Promoter hypermethylation of some genes has been associated with clinical and pathological features of tumor aggressiveness and also with prognostic relevance. Aberrant promoter methylation of *APAF1, DAPK1* and *GREM1* [123] has been associated with aggressive forms of RCC. Moreover, promoter methylation of *APAF1*, *DAPK1* [124], *JUP* [39], *PTEN* [125], *UCHL1* [126], *DAL1-4.1B/EPB41L3* [127] *BNC1* and *COL14A1* [128] have been associated with poorer survival, and most of them (*JUP*, *APAF1*, *DAPK1*, *PTEN*, *DAL1-4.1B*, *BNC1*, and *COL14A1*) retained independent prognostic value in multivariate analysis [39, 124, 128]. Clearly it is important that there should be additional studies of potential methylated biomarkers in tumor tissues and urine and/or blood with the ultimate aim of producing a panel of biomarkers that will enable non-invasive detection, molecular staging and prediction of prognosis. As the number of potential methylated TSG biomarkers increases, it will be of great importance to assay these in a standardized manner in prospective studies to establish their clinical utility.

Genome-wide studies of histone modifications have been performed to characterize the chromatin of malignant cells by establishing the overall profile of histone modifications in cancer cells. Signatures of histone modifications patterns, such as trimethyl-H3K9, are associated with patient prognosis in acute myeloid leukemia [129]. Silencing of genes marked by trimethyl-H3K27 in the absence of DNA methylation has also been reported [130]. Several histone modifications have been associated with poor prognosis in RCC, including low H3K4me2, H3K18ac, and H3K9me2 [131]. H3K4me1–3 levels were also found to be inversely correlated with Fuhrman grade, stage, lymph node involvement and distant metastases, and an H3K4me score was an independent factor for RCC progression free survival [132]. Similar observations have been made for global H3Ac and H4Ac levels, as well as for H3K9Ac levels in RCCs treated with partial nephrectomy [133], whereas H3K18Ac levels were an independent predictor of RCC progression after surgery [134].

The use of genome wide approaches has enabled the production of miRNA fingerprints in a range of tumors and the identification of new potential biomarkers to distinguish tumor tissue from its normal counterpart. From a clinical point of view, miRNAs have great potential as diagnostic and therapeutic agents. Owing to the tissue specificity of miRNAs, they have become a useful tool for defining the origin of tumors in poorly differentiated cancers [135]. Prognosis and survival of patients depends on the cancer stage at diagnosis and miRNA signatures have been reported to be useful tools for early diagnosis of cancer [136, 137]. Differential miRNA expression patterns between neoplastic and non-neoplastic renal tissues, as well as among different renal tumor subtypes have been described. Discrimination between ccRCC and normal kidney tissue have been described with a panel of nine miRs (miR-21, miR-34a, miR-142-3p, miR-155, miR-185, miR-200c, miR-210, miR-224, and miR-592) [Ref. [138]], a combination of miR-141 and miR-155 [139] or by differential expression of miR-92a, miR-210, and miR-200c [140]. For a more clinical perspective with the aim of supporting diagnosis, a stepwise decision tree was created to differentiate between kidney cancer subtypes and oncocytoma, depending on miRNA signatures. This method is valuable in small biopsy samples and in cases where morphological assessment is not sufficient for diagnosis [141]. Unsupervised hierarchical cluster analysis of miRNA microarray data showed that tumors derived from the proximal and distal nephrons can be distinguished by their miRNA profile [140]. The differential expression patterns of miRNAs can also be used to subclassify renal cancer. In ccRCC 23 miRNAs are differentially expressed (let-7e, let-7f, let-7g, miR10b, miR-124, miR-126, miR-138, miR-140-5p, miR-142-5p, miR-144, miR-184, miR-200c, miR-203, miR-206, miR-210, miR-218, miR-27a, miR-27b, miR-335, miR-373, miR-378, miR-92a, miR-98. However, some miRNAs are characteristic of sporadic ccRCC (let-7c, let-7d, miR-1, miR-100, miR-10a, miR-148b, miR-191, miR-199a-3p, miR-19a, miR-215, miR-29b, miR-30c, miR-363, miR-9) and others of hereditary RCC (let-7a, miR-125a-5p, miR-125b, miR-143, miR-146b-5p, miR-15b, miR-17, miR-193a-5p, miR-193b, miR-196a, miR-20b, miR-214, miR-23b, miR-32, miR-372) [61]. miRNA levels in sera of RCC patients and healthy controls, identified miR-1233 as a promising biomarker for RCC detection and monitoring [142]. Altered levels of miRNA might also provide prognostic information. Whereas miR-155 and miR-21 expression in ccRCC tumors has been found to correlate with tumor size [143], higher miR-210 levels were found in tumors displaying higher Fuhrman grade [140]. In ccRCC, overexpression of miR-32, miR-210, miR-21, and miR-18a correlated with poor survival [143, 144]. Lower miR-106b levels were associated with metastatic disease and poorer relapse-free survival [145]. High miR-210 expression was also found in tumors with lymph node metastasis [140], suggesting unique miRNA signatures in metastatic RCC, distinct from those of primary tumors [146]. Khalla et al. [147] compared distant metastases with primary tumors and found a distinct miRNA signature in metastases. Some of the primary tumor samples clustered together with the distant metastasis, suggesting that these primary tumors have a metastasis-specific signature [147]. Because miRNAs can be easily detected and quantified in blood, serum assays based on metastasis-associated miRNAs may be of value.

In addition, Lin et al. [148] identified 12 SNPs in miRNA-related genes that are significantly associated with recurrence or survival and found a cumulative effect of multiple SNPs with recurrence. Taken together, additional studies in large patient cohorts are necessary to validate the potential use of miRNAs as diagnostic/prognostic biomarkers.

4.7 Epigenetics as Consequence or Cause of Cancer Initiation

Cancers are caused by accumulative mutations in the genes [149]. Mutations cause rearrangements of large chromosomal regions, which confer the cells with growth advantage under selection pressure due to abnormal expression of oncogenes [149, 150]. The clonal expansion of the mutated cells leads to genomic instability and global demethylation, while the cell machinery progressively shuts down the anti-survival genes by hypermethylation. Thus mutations cause genomic instability, which precedes methylation changes. By contrast, congenital disorders such as ICF syndrome and Rett syndrome involve genes that encode the methylation machinery of the cell such as *DNMT3B* (ICF syndrome) and *MECP2* (Rett syndrome), but these disorders do not predispose to cancer. Thus, epigenetic changes were thought to be a consequence of altered gene expression rather than causal [151]. Further, activation of tumor suppressor genes by 5-aza-2′-deoxycytidine or *DNMT1* knockout may not be stable, as has been shown for both *MLH1* [152] and *p16* [153], suggesting that the altered methylation might be a consequence rather than a cause of gene silencing. Thus a key barrier to the acceptance of epigenetic alterations as a cause rather than a consequence of cancer has been the lack of well-defined human pre-neoplastic disorders that are caused by epigenetic mutations. However the discovery of the mechanisms of Beckwith-Wiedemann syndrome (BWS) provides a good example of constitutional epigenetic alterations linked to cancer risk. BWS was shown to have various molecular causes, including loss of imprinting (LOI) of *IGF2* [154] or point mutations in the *CDKNIC* [155] gene or epigenetic lesions in the nearby antisense RNA *LIT1*. Furthermore, cancer predisposition might be specifically associated with LOI of *IGF2* and hypermethylation of *H19* [156]. In a large registry of patients with BWS gain of methylation at *H19,* presumably resulting in biallelic expression of *IGF2*, was found to be specifically and statistically associated with cancer risk [157]. BWS leads to an 800 fold increased risk of embryonal tumors such as Wilm's tumor of the kidney and rhabdomyosarcoma [158]. LOI of *IGF2* is specifically associated with increased cancer risk in children with BWS. Thus the epigenetic change precedes cancer and confers risk for cancer, a strong argument for causality. Another study showed that aberrant changes in the epigenome could indeed lead to cancers that do not display genomic instability [159]. *Snf5* is a tumor suppressor gene and a core component of the chromatin remodeling complex SWI/SNF whose inactivation is detected in several types of tumors [160, 161], including the highly invasive

malignant rhabdoid tumors (MRTs) [162]. Differing from most other tumors where the chromosomes are usually fragmented, MRTs often display an intact genome. The authors generated *Snf5*-deficient primary mouse embryonic fibroblasts and showed that tumors derived from these cells were diploid and the cancer phenotype was correlated with the expression of the cell cycle protein cyclin D1, which was epigenetically upregulated by SWI/SNF complexes [159]. An alternative approach to study the relationship between epigenetic changes and transformation is to study the epigenome of pre-cancerous cells. A series of studies on colon cancers found that global hypomethylation as well as regional gene promoter hypermethylation occur in pre-cancerous lesions or even benign colon polyps before they become malignant colon cancers [163–165]. Similar findings have been observed in breast cancers, where normal tissues surrounding the tumors have been detected with aberrant DNA methylation patterns [166, 167]. These observations of methylation patterns change in pre-cancerous cells suggest that the loss in methylation can be an early event that precedes malignancy. Experimental data in mice also support a causal role for epigenetic changes in cancer. When *DNMT1* hypomorphs are crossed with Min (multiple intestinal neoplasia) mice with an *Apc* mutation, they show an increased frequency of intestinal neoplasia and liver cancers [168]. In addition, it has also been shown that global hypomethylation leads to elevated mutation rates [169], suggesting that epigenetic changes may initiate downstream oncogenetic pathways. Studying these model systems may therefore aid our understanding of how epigenetic processes contribute to the process of oncogenic malignancy.

4.8 Epigenetic Therapy

Given that epigenetic modifications are reversible, it seems likely that understanding and manipulating the epigenome may hold promise for preventing and treating common human diseases including cancer. Much attention has been focused on the quest for epigenetic drugs, which restore the normal epigenetic landscape in cancer cells by inhibiting enzymes of the epigenetic machineries. Understanding the mechanisms underlying the tumor suppressor gene silencing in cancer has promoted the idea of pharmacologically relieving the inhibitory effects of DNA methylation and chromatin remodeling on gene expression. Identification of frequently methylated RCC tumor suppressor genes has highlighted potential targets for therapeutic intervention. Decitabine, the clinical form of the demethylating agent 5-aza-2′-deoxycytidine, has been used in several clinical trials, and promising responses have been reported for hematological malignancies such as myelodysplastic syndrome [170, 171]. Various studies have tested DNMT inhibitors or HDAC inhibitors either alone or in combination with conventional chemotherapeutic agents in RCC cell lines with promising results [172–174] but clinical studies are required to conclusively demonstrate the therapeutic usefulness in RCC.

4.9 Conclusions

Understanding the complexity of the epigenome and all the actors involved in modulating its interactions with genomic sequences is of fundamental importance in health and disease. Owing to the reversible and plastic nature of epigenetic alterations, these constitute an attractive target for novel therapeutic intervention. Studying epigenomic alterations and miRNAs provide opportunities for the development of innovative biomarkers to aid in disease detection, diagnosis, prognosis and prediction of response to therapy. Understanding the complex molecular mechanisms involved in epigenetics and miRNAs, may lead to more effective cancer treatments and promote the change from current cytotoxic therapies to more targeted control of malignant phenotypes.

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Chapter 5
Epigenetic Regulation of MicroRNA in Colon Cancer

Qiong Zhang, Mukesh Verma, and Sharad Khare

Abstract Colorectal cancer (CRC) is the leading cause of death from cancer all over the world. New and improved approaches are needed to detect CRC early and develop effective therapeutic approaches. Recently, the role of non-coding small microRNAs (miRs) in CRC initiation and progression has been proposed. In this article, we have summarized which miRs are commonly regulated by epigenetic mechanisms in CRC and their contribution towards gene regulation leading to carcinogenesis. The potential implications of using miR as a marker of risk, therapeutic target and survival are discussed. Future studies should emphasize translational research including the validation of key findings from different laboratories and investigators and the implications of these findings for clinical practice.

Keywords Biomarker • Colon cancer • Epigenetics • Methylation • Micro RNA

5.1 Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008 [1]. Early detection of CRC is needed because the

Q. Zhang • S. Khare, Ph.D. (✉)
Harry S. Truman Memorial Veterans' Hospital, Columbia, MO 65201, USA

Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of Missouri, CE440 Clinical and Educational Support Building, One Hospital Drive, Columbia, MO 65212, USA
e-mail: khares@health.missouri.edu

M. Verma
Epidemiology and Genomics Research Program, Division of Cancer Control and Population Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

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majority of patients who develop CRC have no specific risk factors for the disease and the best indicator of prognosis is based on the stage of the disease. Epigenetic biomarkers, including microRNAs (miRs), may provide tools to detect CRC early as well as opportunities for therapeutic intervention and evaluating survival [2]. Noncoding RNAs, miRs regulate gene expression post-transcriptionally. These miRs are 19– 25-nt noncoding RNAs that are cleaved from 70- to 100-nt hairpin-shaped precursors [3]. Pri-miRs are transcribed in the nucleus and after processing by Drosha and its cofactor DGCR8 transported into the cytoplasm. They undergo processing by the RNAse III enzyme Dicer resulting in the mature miRs, which are incorporated into the RNA-induced silencing complex (RISC). Each step of the process is well regulated, and dysfunction at any level can result in inapt miR functions. To date, more than 1,000 human miRs have been identified and each miR control hundreds of genes [4]. MiRs function as endogenous suppressor of gene expression by binding of RISC to the 3′-untranslated region (3′-UTR) of target mRNAs and inducing either mRNA degradation or translational repression. The mRNA degradation is induced if miR binds completely or almost completely, however, if the binding is incomplete, miR represses translation of mRNA. It has been predicted that miRs regulate the translation rate of more than 60 % of protein coding genes. MiRs have important functions in basic biological processes such as cellular differentiation, proliferation, apoptosis, migration and invasion that constitute the hallmarks of cancer. MiR expression profiles are distinctive between normal tissue and derived tumors and between diverse tumor types. Intriguingly, down-regulation of subsets of miRs is a common finding in many of these studies, indicating that some of these miRs may act as putative tumor suppressor genes. Gene silencing is the most thoroughly studied role of miRs, however, they can upregulate gene transcription during cell cycle arrest [5] and, therefore, overexpression of miRs in human cancers suggested potential oncogenic functions of miRs. In addition, some miRs bind directly to 5′-UTR of target genes and activate rather inhibit gene expression or induce gene expression by binding to the promoter regions [6].

5.2 Aberrant DNA Methylation

The molecular mechanism underlying regulation of miR expression in cancer is not completely understood. Several mechanisms, for example, specific transcriptional regulation, epigenetic mechanisms including methylation and histone deacetylation, mutations affecting proteins involved in the processing and maturation of miRs, or changes in miR stability have been proposed. Cancer cells undergo global DNA hypomethylation [7], which contributes to genome instability. This leads to transcription of silenced transposable sequences, furthering chromosomal rearrangements and genome disruption, a characteristic feature of tumor promotion. Contrary to global hypomethylation, CpG islands of the promoter regions of tumor suppressor genes experience DNA hypermethylation leading to gene silencing and tumor promotion [8]. DNA methylation refers to the covalent addition of a methyl group to the five-position of cytosine usually in a CpG dinucleotide in differentiated cells. Epigenetic mechanisms accounting for the observed down-regulation of tumor suppressive miRs

in human cancer are CpG island hypermethylation or histone modifications in a similar manner that is now well accepted for classic tumor suppressor genes. MiRs that are transcribed from CpG islands undergo DNA hypermethylation-coupled repression due to binding of the transcriptional repressor methyl CpG binding proteins and histone modifications such as loss of acetylation of histones H3 and H4 [9]. About 16 % of the annotated human miRs are located within 1,000 bp of a CpG island and thus epigenetic regulation of miRs might be more common than reported so far.

5.3 Epigenetically Silenced miRs in Colon Cancer

The list of miRs that undergo CpG island promoter hypermethylation in CRC is growing rapidly [10, 11]. A recent epigenomic survey of 450,000 CpG sites revealed that 30–40 miRs underwent hypermethylation in CRC cells [11]. Epithelial-mesenchymal (EMT) and mesenchymal-epithelial (MET) transitions occur in tumorigenesis. EMT is a complex process that includes disbanding of cell-cell junctions, and loss of apicobasal polarity, which gives rise to migratory and invasive mesenchymal cells. During this process loss of adhesion proteins such as E-cadherin and γ-catenin and gain of mesenchymal proteins such as Vimentin and N-cadherin is invariably observed. The role of E-cadherin repressors such as SNAIL, ZEB1, ZEB2 and E47 is very well established. Once metastasized, mesenchymal cells require MET conversion for proliferation of dispersed cancer cells at secondary locations. It has been suggested that methylation dependent regulation of miR-200 family plays a crucial role in EMT and MET [12]. The putative DNA methylation regulated inactivation of various miR-200 members has been described in cancer [13]. The miR-200 family is consists of five members that can be divided into two clusters: miR-200a/b/429 on chromosome 1 and miR-200c/141 on chromosome 12. The miR-200 family is recognized as a key regulator of the epithelial phenotype by targeting ZEB1 and ZEB2, two important transcriptional repressors of the E-cadherin (cell adherence) and *CRB3* and *LGL2* (polarity) genes. The miR-200a/b/429 and miR-200c/141 transcripts endure an active epigenetic regulation linked to EMT or MET phenotypes in tumor development. The 5′-CpG islands of both miR-200 loci are normally unmethylated and linked to epithelial features, such as low expression of ZEB1/ZEB2 and high levels of E-cadherin, CRB3 and LGL2. However, 5′-CpG island hypermethylation-associated silencing is observed in transformed cells with mesenchymal characteristics. The stable transfection of miR-200a/b/429 and miR-200c/141 in the hypermethylated cells reinstated the epithelial markers with inhibition of cell migration, tumor growth and metastasis in nude mice. Furthermore, the miR-200 epigenetic silencing is a dynamic process, which can be shifted to hypermethylated or unmethylated 5′-CpG island status corresponding to the EMT and MET phenotypes, respectively. Laser micro-dissection revealed that normal colonic crypts (epithelia) and stroma (mesenchyma) are unmethylated and methylated at these loci, respectively. However, the colorectal tumors undergo selective miR-200 hypermethylation in epithelia during EMT. These findings signify that the epigenetic manipulability of the miR-200 family contributes to the adapting phenotypes of CRC [13]. Other studies also

demonstrated that DNA methylation dependent silencing of miRs is involved in the metastatic phenotype [14]. Treatment of cancer cells with DNA methylation inhibitor exhibited cancer-specific hypermethylation of the CpG islands in the promoter regions of miR-148a, miR-9 family and miR-34b/c [15]. Mir-34 family, tumor suppressor miR family, is regulated by transcriptional factor p53 and DNA methylation [16]. Metastatic genes such as c-Myc, cyclin-D kinase-6 (CDK6) and E2F3 are target genes of miR-34b/c whereas TGIF2 is target of miR-148a.

A recent study first identified 23 miRs that are down-regulated in human primary CRC compared with matched normal colorectal epithelium [17]. Of the 23 miRs down-regulated in CRC, 5 of them miR-9, miR-124, miR-129, miR-137 and miR-149 are located with-in 1,000 bp of a CpG island. Treatment with DNA methyltransferase and HDAC inhibitors restored the expression of 3 of the 5 miRs in 3 colon cancer cell lines. Subsequently it was shown that miR-9-1, miR-129-2 and miR-137 genes were silenced in CRC and this was mediated at least in part by epigenetic mechanisms including DNA hypermethylation and histone acetylation [18]. After treatment with HDAC inhibitor, CRC cell lines were subjected to chromatin immunoprecipitation with an antibody against acetylated H3 (a mark of transcriptional active chromatin) followed by Q-PCR. Results demonstrated an increase in AcH3 in the case of miR-9-3 and miR-127 but no significant changes in miR-9-1, miR-129-2 and miR-137. These results indicated that expression of mature miR-9 could be regulated by hypermethylation of miR-9-1 locus and H3 de-acetylation of the miR-9-3 locus [18]. The association between miR-9-1 methylation and more advanced stages and nodal involvement suggested a role of miR-9-1 as a potential tumor marker of poor prognosis in CRC. However, the precise functions and targets of these miRs in CRC were not investigated [18]. Another study also exhibited that miR-137 is hypermethylated in the early stages of CRC and targets CDK6, lysine-specific histone demethylase-1 (LSD-1) and E2F6, a transcription factor, which is involved in cell cycle [19].

Intronic regions of coding genes can also be transcribed into miRs and this may also explain that tumor phenotype is due to the hypermethylation of a gene that in turn down regulates miR it harbors. The example of this scenario is miR-342 that is transcribed from EVL gene. The EVL gene affects cell polarity, cytoskeleton remodeling and cell motility. The EVL promoter is hypermethylated in the early stages of CRC and thus the gene and miR-342 both are downregulated [20]. The EVL/miR-342 locus is methylated in 86 % of colorectal adenocarcinomas and in 67 % of adenomas, indicating that it is an early event in colorectal carcinogenesis.

The genetic disruption by homologous recombination of DNA Methyltransferase1 (DNMT1) and DNMT3b (DKO, double knock out) of wild type colon cancer cell line HCT-116 showed a drastic reduction of DNMT activity, 5′-methylcytosine DNA content, and, most significant, a release of gene silencing associated with CpG island hypermethylation [9]. Further, the comparison of miR expression profile of HCT-116 wild type cells with DKO cells established that epigenetic silencing of the tumor-suppressor miR-124a by aberrant DNA hypermethylation leads to CDK6 overexpression a bona fide oncogenic factor [9]. Transfection of the tumor cell line with miR-124a reduces CDK6 protein levels and retinoblastoma (Rb) phosphorylation, one of the targets of CDK6 [9]. Rb, an antiproliferative tumor-suppressor gene when phosphorylated by CDK6, abrogates its antiproliferative activity.

A recent study identified silencing of DNA methylation-regulated miR-941, miR-1237 and miR-1247 in CRC cells [21]. Ectopic expression of miR-1247 significantly reduced cancer cell proliferation and migration in HCT-116 and DLD-1 cells suggesting that miR-1247 may work as tumor suppressor. Several predicted targets of miR-1247 were proposed, for example, Citron (CIT), a serine threonine kinase that regulates G2/M transition in cell cycle, FosB that dimerizes with Jun protein to activate transcription and transmembrane glycoprotein ADAM15, which has a role to play in cancer metastasis. These computationally predicted targets need validation in future studies. Overexpression of miR-941 significantly inhibited cell migration in both HCT-116 and DLD-1 cells. Predicted target for miR-941 was metallopeptidase24 (MMP24), which facilitates tissue remodeling and cell migration [21].

Treatment of colon cancer cell lines with 5-aza-2′-deoxycytidine, a methylation inhibitor resulted in greater than two-fold increased abundance of 10 miRs [22]. Amongst them transfection of miR-373 in colon cancer cells resulted in decreased cell proliferation. In patient biopsies, miR-373 was down regulated in colon cancers (29/40, 72.5 %) relative to control samples, whereas a member of Ras oncogene family RAB22A, predicted target gene of miR-373 was upregulated (24/40, 60 %). Aberrant methylation of miR-373 in colon cancers (35/40, 87.5 %) relative to controls (8/40, 20 %) was also noted [22]. These studies established that one mechanism accounting for the transcriptional down regulation of miRs in human cancer is CpG island hypermethylation.

5.4 Methods

The mirVana RNA isolation (Ambion, Texas) or Exiqon miRNA isolation kits are frequently used for miR isolation and enrichment. TaqMan real time QPCR for miR quantification and microarrays are commonly used for miR profiling. The gold standard method to evaluate the methyl status of cytosine residues is bisulfite sequencing. For this DNA is treated with sodium bisulfite (CpGenome DNA modification kit) and then sequenced using primers specific to bisulfite sequence. These methods, routinely used in miR research, are discussed in detail earlier [23]. Mature miR mimics are transiently transfected or miR genes are stably transfected to increase miR expression levels in cancer cells. To confirm target genes of miR, luciferase vectors (empty luciferase vector or luciferase vector containing wild-type or mutant-type target gene 3′-UTR) are transfected for luciferase reporter assays.

5.5 Translational Applications and Future Directions in MiR Research

The DNA methylation profile of miRs can delineate tumor type, clinical prognosis and treatment response. miRs have great potential at the diagnostic and therapeutic levels. miRs circulate in blood stream in a stable form and thus can function as early

biomarkers in a clinical setting. More than 100 studies have been reported so far to assess the potential role of serum and plasma miRs as biomarkers for different types of cancer [24]. To improve CRC diagnosis, use of miR DNA methylation in biological fluids such as colorectal mucosal wash fluids [25] and feces [26] has been described. On the basis of clinical trials, DNA methyltransferase inhibitor azacytidine (Vidaza™) was approved for the treatment of myelodysplastic syndrome. Azacytidine is being evaluated in clinical trials for other malignant diseases. Similarly, Vorinostat (Zolinza™) and Romidepsin (Isotodax™) both novel HDAC inhibitors have been approved for the treatment of refractory cutaneous T cell lymphoma [24]. More studies are required to find out whether these epigenetic drugs would modulate miR expression in colon cancer.

Information about miRs profiling and/or single nucleotide polymorphism (SNP) have been used in colon cancer epidemiology studies [27–32]. For example, high miR-21 expression in colon adenocarcinoma was associated with a low survival rate and resistance to therapy in 196 patients [32]. Another study demonstrated role of miR-106a in prediction of survival of 110 colorectal cancer patients [27]. The prognostic value of miR-215 was noted in stage II and III of colon cancer when patient's samples were analyzed by quantitative PCR [28]. MiR-143 was noted as a novel prognosticator and a promising drug target in 77 colorectal patients with KRAS mutations and undergoing EGFR targeted therapy with the monoclonal antibodies cetuximab or panitumumab [30].

A SNP rs2737 in the IC53 gene (gene associated with the grade and depth of adenocarcinoma) affected expression of miR-379, and this polymorphism was found to be associated with late onset of colorectal cancer in 222 patients [33]. Thus, miRs may have role in cancer protection. However, case–control study (Central-European Caucasian population) evaluated SNPs in miR-196a, miR-27a, and miR-146a and their association with CRC, but no significant association was observed [34]. Polymorphism rs4919510 in miR-608 was associated with CRC outcome but not with risk [35]. MiRs usually regulate gene expression by binding to the 3′-UTR of mRNAs. Due to the stringent recognition requirement needed by the miR and the binding region on its target gene, it is quite conceivable that SNPs could have functional implications on the post-transcriptional regulation of target genes. A SNP could either weaken a known miR target or create a sequence match to the miR that was not previously associated with the given mRNA. Changes in the expression pattern of a gene could therefore influence a person's risk of disease.

Altered miR expression is a hallmark of a number of tumor types including CRC. Hundreds of miRs have been identified to date; however, computer models suggest there may be thousands more. As bench-work continues to verify *in silico* predictions, miR profiling will remain a prominent tool for identification of differentially expressed miRs in normal cellular courses and human disorders including CRC. Now, the results should be validated in a large number of samples collected from a variety of geographically dispersed populations. As with other cancers, screening of populations at high risk of developing CRC, using miR profiling might be the first step towards preventing these cancers. Emphasis should be placed on the identification of polymorphisms in the double-stranded region of CRC specific miRs. Since the cost of high throughput sequencing has decreased significantly, this objective is now

Table 5.1 Selected miRs in colon cancer and their characteristics

Micro RNAs	Characteristics
miR-1	Under-expressed [36]; inhibits cell proliferation and viability [37]
miRs-122, -214, -372, -15b, let-7e, -17	Differentially expressed and affect p53 pathway [38]
miR-195	Down-regulated in CRC and correlates with lymph node metastasis and poor prognosis [39]
miRs-15b, -181b, -191, -200c	Over-expressed [40]
miR-499	Under-expressed [41]
miR-9-1	Expression is inversely associated with its promoter methylation; associated with lymph node metastasis [18]
miR-21	Acts as an oncomiR; inflammation-mediator in CRC [42]; interacts with PTEN/PI-3 K/Akt signaling pathway [43]; over-expressed in high-risk stage II CRC patients [44]
miRs-34a, -34b/c	Inactivation due to promoter methylation [45]; in Wnt-signaling [46]; regulate Axl receptor expression [47]
miR-92	Higher levels in adenomas and carcinomas than other miR-17-92 cluster members (miR-17, miR-18a, miR-19a, miR-19b, miR-92a) [48]
miRs-31, -223	Over-expressed in CRC of patients with hereditary non-polyposis colorectal cancer syndrome (Lynch Syndrome) [49]
miRs-192, -215, -26b, -143, -145, -191, -196a, -16, let-7a	Under-expressed in CRC [49]
miRs-31, -183, -17-5p, -18a, -20a, -92	Over-expressed in CRC [50]
miR-135b	Correlated with the degree of malignancy [51]
miRs-105, -549, -1269, -1827, -3144-3p, -3177, -3180-3p, -4326	Upregutaed in CRC [52]
miR-126	Under-expressed in CRC [53]
miR-129	Regulates cell proliferation; interacts with Cdk6 [54]
miRs-17-92 cluster, miRs-21, -135	Could be detected in exfoliated colonocytes isolated from feces for CRC screening; upregulated in CRC [55]; interaction of miR-135 with APC expression and Wnt pathway [56]
miRs-182, -17, -106a, -93, -200c, -92a, let-7a, -20a	Upregulated in CRC [57]
miRs-215, -375, -378, -422a	Decreased in CRC [58]
miRs-127-3p, -92a, -486-3p, -378	Down-regulated in KRAS mutation positive samples [59]
miR-200c	Involved in epithelial to mesenchymal transition [12]

achievable and few reports are already available [34, 35]. For future research, we should take into account miRs and their regulatory networks in order to understand the complex processes underlying malignant transformation. Additional research investments are needed in this area of science (Table 5.1).

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Chapter 6
Epigenetic Regulation of miRNAs in Breast Cancer Formation and Progression

Zhiqian Zhang, Zhengmao Zhu, Baotong Zhang, and Jin-Tang Dong

Abstract Breast cancer is a leading cause of cancer death among women. Breast cancers include diverse subtypes, complicated cellular and molecular characteristics, a large number of genetic alterations, and a broad range of clinical behaviors. Great advances in cellular and molecular biology have shed new light on the roles of various genes in the formation and progression of breast cancer. Whereas somatic and germline abnormalities occur in many of these genes during breast carcinogenesis, it has become clear that epigenetic abnormalities also contribute to breast cancer development. For example, hypermethylation of promoter DNA is a common mechanism for the downregulation and loss of function of tumor suppressor genes, and abnormalities in chromatin modification dysregulate both tumor suppressor genes and oncogenes during the development and progression of breast cancer. microRNAs (miRNAs), a class of small, highly conserved endogenous non-protein-coding RNAs, downregulate their target genes to impact multiple biological processes including tumorigenesis, and thus represent another mechanism of epigenetic regulation of genes. Interestingly, a number of miRNAs are commonly dysregulated in breast cancer by different mechanisms including epigenetic mechanisms such as promoter methylation and histone modification. Furthermore, abnormal expression of miRNAs can also modulate the epigenetic profiles of cells. This chapter will

Z. Zhang • J.-T. Dong (✉)
Department of Genetics and Cell Biology, Nankai University College of Life Sciences, 94 Weijin Road, Tianjin 300071, China

Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University School of Medicine, 1365 Clifton Road, Atlanta, GA 30322, USA
e-mail: j.dong@emory.edu

Z. Zhu • B. Zhang
Department of Genetics and Cell Biology, Nankai University College of Life Sciences, 94 Weijin Road, Tianjin 300071, China

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focus on the influence of epigenetic changes on the expression of miRNAs in breast cancer formation and progression. We will also review the epigenetic events that are regulated by miRNAs in breast cancer.

Keywords miRNAs • Breast cancer • Epigenetic regulation • Methylation • Acetylation

6.1 Introduction

MicroRNAs (miRNAs) are a family of small (usually about 19–24 nucleotides (nt) in length), endogenous, non protein-coding RNAs that downregulate gene expression by binding to complementary sites on the 3′ untranslated region (3′ UTR) of specific target mRNAs, which causes target mRNA cleavage (mainly in plants) and/or inhibition of protein synthesis (mainly in animals). miRNAs are often transcribed by RNA polymerase II as large primary miRNAs (pri-miRNAs), which may be either translated into proteins or processed by the RNase III Drosha to about 70 nt hairpin-structured secondary precursors named pre-miRNAs. After being transported from the nucleus to the cytoplasm by exportin-5, the pre-miRNAs are then processed to mature miRNAs by another RNase III, Dicer, and its partners. Since they were first discovered in the nematode *Caenorhabditis elegans* as important regulators of developmental timing, miRNAs have been found to be extremely conserved and represent a crucial layer of RNA-based gene regulation both in plants and animals. miRNAs participate in almost all important biological processes including cellular proliferation, differentiation, senescence, apoptosis, stem cell maintenance, hematopoiesis, metabolic homeostasis, defense against viruses and organic development. Their dysregulation is also highly related to some pathogeneses such as diabetes, heart diseases, cardiovascular disease, autoimmune disease, mental disorders, as well as tumorigenesis. miRNAs and their targets form a sophisticated network that plays a pivotal role in tumor formation and progression.

Breast cancer, a heterogeneous disease with diverse subtypes, complicated cellular and molecular characteristics, multiple genetic alterations, and diverse clinical behavior, is the second leading cause of cancer-related deaths in American women, and tumor metastasis is responsible for the vast majority of cancer-related deaths. During the past several decades, advances in understanding the molecular and cellular basis of breast cancer development, especially in metastasis, have been well documented. Based on currently available evidence, miRNAs widely participate in the formation and progression of most types of cancers including breast cancer. More than half of all human miRNA genes are located in fragile sites of the human genome, which are associated with tumors of different origins. The role of miRNAs in breast cancer has been extensively investigated. As one of the major factors in epigenetic regulation of biological processes, miRNAs can function as oncogenes or tumor suppressor genes. For example, some miRNAs (miR-21, miR-224, miR-155,

miR-135a, miR-10b, miR-373 and miR-520c) have been reported to be oncogenes while other miRNAs (miR-206, miR-30a, miR-125a, miR-125b, miR-34 and miR-31) have been reported to suppress tumor development and invasion in breast carcinoma. In addition, several miRNAs are related to particular molecular characteristics, tumor subtypes, or specific tumor stages.

Abnormal epigenetic modifications are critical for breast cancer formation and progression. In addition to DNA methylation and histone modification, miRNA-mediated gene regulation is another common epigenetic modification. However, miRNA regulation and other epigenetic modifications are not entirely separable, as miRNAs are also regulated by other epigenetic modifications in their transcription. For example, DNA methylation can silence the transcription of miRNAs in different types of cancers including breast cancer. Investigation of the mechanisms by which epigenetic changes control miRNA expression could lead to a better understanding of breast cancer formation and progression and improved diagnosis and treatment of patients with breast cancer.

6.2 miRNA and Breast Cancer

Many studies have described, and several reviews have summarized, the association between miRNA dysregulation and the development and clinical characteristics of breast cancer. Here we will briefly review those miRNAs that have been identified with important functions and genetic alterations.

6.2.1 miRNA Dysregulation Contributes to Breast Tumor Formation and Progression

Genes for miRNAs are often located in fragile sites of human chromosomes, which undergo frequent genetic alterations such as gene mutation and chromosomal deletion and amplification. In 2005, genome-wide miRNA expression profiling in breast cancer specimens and normal tissues identified 29 miRNAs that are abnormally expressed in breast cancer [1]. Most of these miRNAs have been shown to play crucial roles in breast cancer in later studies. For instance, let-7 family members, which were originally discovered for their impact on the development of *C. elegans*, were found to be downregulated in breast tumor-initiating cells (BT-ICs), and their enforced expression in BT-ICs reduced their proliferative capability and their ability to form mammospheres and tumors [2]. Other miRNAs are downregulated in breast cancer and could therefore be tumor suppressors. For example, miR-34, which is downregulated in several human malignancies including breast cancer, has been shown to be regulated by p53 as an important effector of p53 function [3]; miR-101 is downregulated via genomic loss, and its downregulation during breast

cancer progression is accompanied by an increase in the expression of EZH2, an oncogenic transcription factor [4]. Some miRNAs, including miR-125a and miR-125b, are specifically downregulated in HER2-positive breast cancers, and their expression could reduce Her2 and Her3 protein levels and suppress anchorage-dependent growth, cell motility and cell invasion [5]. miR-203, a skin miRNA modulating epithelial differentiation, suppresses breast cancer invasion and migration by targeting to SNAI2 [6].

Many miRNAs also promote breast cancer when upregulated. For example, miR-10b is highly expressed in metastatic breast cancer cell lines, and its overexpression triggers tumor invasion and distant metastasis by suppressing the translation of HOXD10, a gene inhibiting the expression of several genes involved in cellular migration and extracellular matrix remodeling [7]. Another miRNA consistently upregulated in many types of cancers, miR-21, could promote breast cancer proliferation and inhibit apoptosis by targeting Bcl2 and PDCD4 [8]. In addition, miR-155 is highly expressed in aggressive breast tumors to mediate epithelial mesenchymal transition (EMT) induced by TGF-β/Smad4 signaling [9], and miR-206 is upregulated in estrogen receptor (ER)-positive breast cancer to downregulate ER protein expression [10].

miRNAs show great promise as clinical biomarkers for breast cancer classification and diagnosis. Despite modulating the expression of protein-coding genes in cells, more and more tumor-associated miRNAs have been found in extracellular tissues including serum, urine, plasma and other body fluids. These so-called circulating miRNAs are highly stable and fairly easy to detect in serum and plasma. The expression profile of circulating miRNAs is also usually highly specific to a specific disease including breast cancer. Therefore, expression patterns of circulating miRNAs, particularly serum miRNAs, could be used as novel biomarkers for diagnosing and monitoring individual breast cancers.

6.2.2 miRNA Dysregulation by Genetic Mechanisms

Genomic alterations represent one of the most important factors that dysregulate miRNA expression during cancer development. For example, the miR-17-92 cluster is located in a chromosome region at 13q31 that is frequently amplified in cancers. Indeed, genomic amplification and overexpression of miR-17-92 have been detected in several human B-cell lymphomas, and miR-17-5p was found to be upregulated in human breast cancer and to promote migration and invasion of human breast cancer cells via the suppression of HBP1 [11]. On the other hand, miRNAs can be downregulated by chromosomal deletion, as seen for the miR-15a and miR-16-1 cluster, which is located at a region at 13q14 that is often deleted in leukemia and solid tumors including breast cancer. These miRNAs could be upregulated by curcumin, an anticancer agent, in MCF7 cells, and induce apoptosis by suppressing Bcl2 expression [12].

Genetic alterations can also modulate the biogenesis of miRNA in breast carcinogenesis. The gene for expotin 5, which is responsible for exporting pre-miRNAs from the nucleus to the cytoplasm, contains two missense SNPs in its coding region, rs34324334 and rs11544382. SNP rs11544382 is associated with an increased risk of breast cancer [13]. Additional genetic polymorphisms of genes associated with miRNA biogenesis have been shown to be associated with disease-free survival and/or overall survival among 488 breast cancer patients, including SNPs rs11780640 and rs2292779 in AGO2, SNP rs1057035 in DICER1, SNPs rs4759659 and rs11060845 in HIWI, SNP rs9606250 in DGCR8, SNP rs874332 in DROSHA, and SNP rs4968104 in GEMIN4 [14]. These findings suggest that molecular modification involved in miRNA biogenesis can influence breast carcinogenesis by regulating miRNA biogenesis and transportation.

6.3 Epigenetic Abnormalities and Breast Cancer

Epigenetic modifications are stable, flexible and inheritable, and they represent a common mechanism for gene regulation without altering DNA sequences. Epigenetic modifications include DNA modification, histone modification, non-coding RNA regulation, nucleosome remodeling, and higher-order structural chromosome arrangement. Understanding the relationship between epigenetic alterations and cancer allows for better clinical diagnosis and improved therapies for cancer patients. Although the details of epigenetic modifications and their roles in breast cancer remain largely undetermined, available findings indicate that different modifications influence breast cancer development, and that they are often closely interconnected.

6.3.1 DNA Methylation and Breast Cancer

DNA methylation is a type of covalent modification in which a methyl group is added to the 5-carbon on a cytosine residue in the genomic DNA by DNA methyltransferases (DNMTs) with S-adenosylmethionine as the methyl donor, and this enzymatic reaction often occurs after DNA replication. In mammalian somatic cells, DNA methylation is almost restricted to CpG dinucleotides, which are usually clustered in about 0.5–3 kb chromosome regions so called CpG islands. CpG methylation suppresses gene expression by inhibiting the access of the transcription factor to their binding sites. DNA methylation is the earliest and the most extensively described epigenetic alteration in carcinomas. Approximately half of human gene promoters contain CpG islands, which are usually unmethylated in normal cells but often hypermethylated in cancer cells, and the hypermethylation patterns are tumor-type specific. During breast cancer development and progression, a number of tumor suppressor genes such as ANKRD11, BRCA1, CCND2, CDH1, CDH13,

CHD5, ERα, ERβ, GSTP1, HOXA5, PITX2, RAR, and RASSF1A have been reported to be hypermethylated. BRCA1 is a well known tumor suppressor gene involved in the maintenance of genome integrity, and its germline mutations exist in almost half of hereditary breast carcinomas. Hypermethylation of the *BRCA1* promoter contributes to the inactivation of *BRCA1* in 7–31 % of sporadic breast and ovarian cancers [15]. DNA hypomethylation also frequently occurs in breast cancer, mainly in repeated DNA sequences and pericentromeric satellite DNA and sometimes also in protein-coding genes. DNA hypomethylation is often necessary but not sufficient for gene expression, and its effects in increasing expression of target genes require the involvement of some transcription factors [16]. In mammals, there are five DNMT members (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L), and only three of them (DNMT1, DNMT3A and DNMT3B) seem to have catalytic methyltransferase activity. In primary breast tumors and breast cancer cell lines with basal-like subtype, DNMT3b overexpression significantly contributes to elevated DNMT activity and induces a hypermethylated phenotype [17]. Meanwhile, a number of bioactive agents and dietary compounds can modulate the enzymic activity of DNMTs to change the methylation patterns of gene promoters in breast cancer [18]. New advances have been made in techniques that can detect genome-wide DNA methylation and some have been utilized to study the methylation status of the breast cancer genome.

6.3.2 Histone Modification and Breast Cancer

Another frequent epigenetic modifying event is histone modification. Histones are a family of alkaline proteins in eukaryotic cell nuclei that form nucleosomes with DNA. Two of the core histones (H2A, H2B, H3, and H4) assemble to form one octameric nucleosome core particle wrapped with 147 bp DNA. The tail of histones can be reversibly modified posttranslationally by methylation, phosphorylation, acetylation, ubiquitination, sumoylation, and ADPribosylation, and these modifications can alter the accessibility of gene promoters and enhancers for various transcriptional activators and repressors in gene transcription. Aberrant changes in the levels of histone modification are frequently observed independent of or concurrent with DNA hypermethylation in breast cancer. Understanding the mechanisms of abnormal histone modifications and their contribution to breast cancer development and progression is critically important for the development of novel targeted therapy for breast cancer patients. For example, inhibition of histone deacetylase (HDAC) activity by specific HDAC inhibitors reactivates estrogen receptor (ER) expression by reorganizing heterochromatin-associated proteins without alteration in promoter DNA hypermethylation in ER-negative breast cancer cells. Inhibition of the class III HDAC SIRT1 also reactivates silenced tumor suppressor genes without loss of promoter DNA hypermethylation in breast cancer cells [19]. Histone methylation occurs in a more complicated manner than histone acetylation in breast cancer. Overexpression of the polycomb group protein EZH2, a methyltransferase

essential for the epigenetic maintenance of H3K27 methylation, has been reported as a marker of aggressive breast cancer [20]. Histone lysine-specific demethylase 1 (LSD1) is highly expressed in ER-negative breast cancer cells and inhibits the invasion of breast cancer cells *in vitro* and suppresses breast cancer metastatic *in vivo* [21]. Upregulation of SMYD3, a histone H3K4-specific methyltransferase, plays a key role in the proliferation of breast cancer cells [22].

6.4 DNA Methylation Regulates miRNA Expression in Breast Carcinoma

Epigenetic regulation including DNA methylation plays an important role in the regulation of miRNA expression. Aberrant methylation of miRNA promoters dysregulates miRNA expression and could be an important early event during tumor formation. miRNA genes whose promoters are targeted by the polycomb complexes are more prone to hypermethylation in breast cancer cells. Inactivation of miRNAs due to genetic deletion or mutation has been well described and reviewed in human malignancies. Here, we will review the role of aberrant hypermethylation as an additional mechanism for miRNA inactivation in human breast carcinoma (Figs. 6.1 and 6.2; Table 6.1).

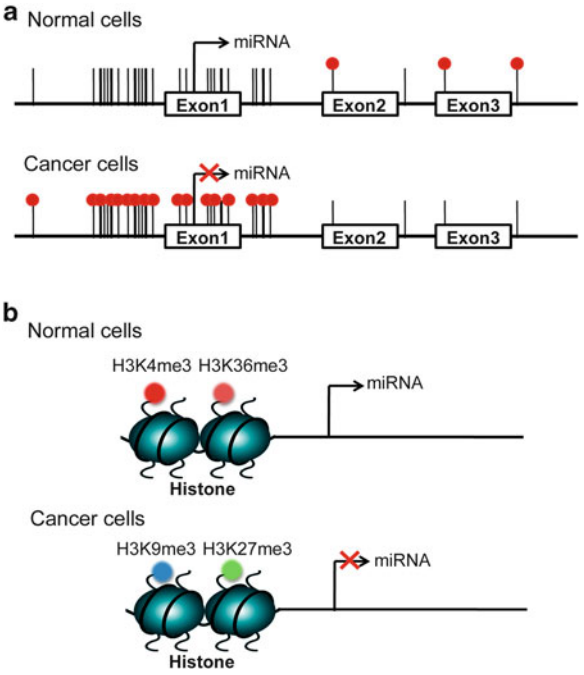

Fig. 6.1 Differential regulation of miRNAs by epigenetic mechanisms in normal and cancer cells. (**a**) Hypermethylation of gene promoters in cancer cells downregulates miRNA expression. *Horizontal lines* indicate promoter DNA, where CpG dinucleotides are marked by *vertical lines* and methylated CpG by *red dots*. (**b**) Alterations in the methylation of histone tails on nucleosomes also cause abnormal expression of miRNAs in breast cancer cells

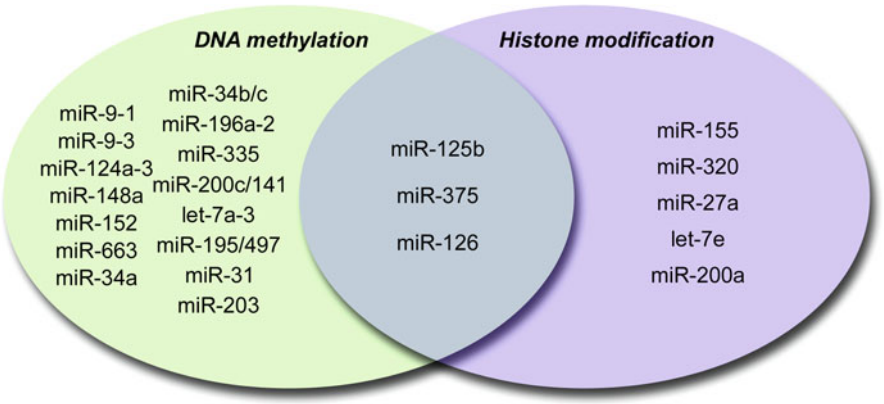

Fig. 6.2 Venn diagram showing miRNAs that are regulated by DNA methylation, histone modification or both

6.4.1 DNA Hypermethylation Leads to miRNA Dysregulation in Breast Cancer

In the past few years, studies on the role of DNA methylation in miRNA expression during breast cancer development and progression have grown rapidly. Like the methylation of protein coding genes, individual miRNA genes are also methylated in breast carcinoma. Lehmann et al. [24] first described DNA methylation as an additional mechanism for miRNA silencing. They first identified *in silico* 61 miRNA genes with CpG islands in their promoters and then assessed the methylation status of these miRNA genes in five breast cancer cell lines and different normal tissues. Twelve of the miRNA genes showed moderate to high levels of methylation in at least two breast cancer cell lines but not in normal tissues. Among those 12 miRNA genes, five (miR-9-1, miR-124a3, miR-148a, miR-152, and miR-663) were strong hypermethylated not only in cell lines but also in 20 primary tumors of breast cancer. They further found that the quantitative methylation status of these miRNA gene promoters was highly correlated with some highly methylated genes examined in their previous studies. When treated with the demethylating agent 5-aza-2′-deoxycytidine in breast cancer cell lines BT-474, EFM-19, SK-BR-3 and ZR-75-1, the miR-9-1 gene was demethylated and upregulated while four other miRNA genes showed no demethylation and/or upregulation. Hsu et al. [25] also reported aberrant DNA hypermethylation of miR-9-3, which is located in a different chromosome region than miR-9-1, in mammosphere-derived breast epithelial cells that had been exposed to a synthetic estrogen named diethylstilbestrol which involved the recruitment of DNMT1. MiR-9-3 is a potential hallmark for early breast cancer formation, and restoration of its expression by the combination of a demethylation agent and miRNA-based therapies may be a reasonable option for patients with early stage breast cancer.

Table 6.1 List of miRNAs that are downregulated by promoter methylation in breast cancer

MiRNA	Target(s) identified	Functional notes	References
Let-7a-3	ERα, caspase 3, H-RAS, HMGA2	Regulates ER signaling. Suppresses therapy-induced cell death. Regulates self renewal and tumorigenicity of cancer cells	[23]
miR-9-1	MTHFD2, E-cadherin	Inhibits cell proliferation and metastasis. A potential biomarker for recurrence and ER status	[24]
miR-9-3			[25]
miR-31	β1-integrin, RhoA	Inhibits cell spreading and metastasis	[26]
miR-34a	Bcl2, SIRT1, Snail1, Axl, β-catenin	Snail1-dependent EMT. Suppresses Wnt signaling. Inhibits cell proliferation and migration	[27]
miR-34b			[27]
miR-34c			[27]
miR-125b	STARD13, ARID3B, ETS1, Mucin1, Bak1, C-Raf, Her2, ESR1	Enhances cell proliferation and tumor metastasis. Confers resistance to chemotherapy	[28]
miR-124a-3	IQGAP	Suppresses multiple steps of metastasis. Mutation predisposes to cancer	[29]
miR-141			[30]
miR-148a			[24]
miR-152			[24]
miR-195	Raf-1, Ccnd1	Inhibits tumor growth. A potential biomarker for noninvasive and early stage disease	[31]
miR-196a-2	HOXC8	Suppresses cancer cell migration and metastasis	[32]
miR-200c	Snail1, ZEB1/2, SIRT1, Suz12, Sec23a, PLCγ1	Suppresses EMT, resistance to apoptosis, and breast cancer stem cell phenotype	[30]
miR-203	SLUG/Snail2, BIRC5, LASP1, SOCS3	Suppresses cell proliferation and migration. Enhances chemosensitivity	[6]
miR-335	SOX4	Inhibits lung metastasis	[33]
miR-375[a]	RASD1	Drives the proliferation of ER+ breast cancer cells. Regulates ER signaling	[34]
miR-497			[31]
miR-663			[24]

[a]Indicates an upregulation in ER-positive breast cancer cells

The miR-34 family (miR-34a and miR-34b/c) are important factors in the p53 tumor suppressive network, directly transactivated by p53. The three members of the family are encoded by two independent genes: the gene on 1p36 for miR-34a and the gene on 11p23 for miR-34b and miR-34c. Both genes have a CpG island in their promoter regions. The miR-34 family possesses tumor suppressive properties such as apoptosis induction, cell cycle arrest, senescence induction, and migration suppression in human malignancies. These miRNAs are frequently inactivated in breast, ovarian, lung, colorectal, bladder and pancreatic carcinomas. The miR-34 family is downregulated in breast cancer cell lines derived from triple-negative tumors, which have a higher incidence of p53 mutations, and the inactivation of miR-34a may be mediated by CpG hypermethylation within the region 100–500 bp upstream to the miR-34a transcription start site that contains a p53 binding site. In addition, genomic deletion including loss of heterozygosity (LOH) in 1p36 could also contribute to the silencing of miR-34a in breast cancer [35]. More interestingly, miR-34c expression is reduced in BT-ICs, and even a single hypermethylated CpG site in its promoter region could lead to its transcriptional inactivation [36]. This single site hypermethylation influences miR-34c transcription by decreasing the binding activity of transcription factors, and downregulation of miR-34c promotes self-renewal and EMT of BT-ICs. Frequent downregulation of miR-34a and miR-34b/c by CpG methylation has also been detected in formalin-fixed, paraffin-embedded tissues of multiple types of cancers including breast cancer.

The expression of the same miRNA can be modulated by both genetic variation and epigenetic promoter methylation. After screening for genetic variants (single nucleotide polymorphisms, SNPs) of 15 miRNAs in breast cancer, Hoffman et al. [32] found a significant association between a common sequence variant (rs11614913, C→T) of miR-196a-2 and reduced breast cancer risk. Meanwhile, the CpG island hypermethylation of the miR-196a-2 precursor occurred in breast cancer and was also associated with decreased breast cancer risk. Examination of different alleles of the miR-196a-2 precursor in the breast cancer cell line MCF-7 suggested that the mutant T allele leads to a greater decrease in expression of mature miR-196a than the common C allele [32] in addition to slowing cell cycle arrest [32], suggesting that miR-196a plays an oncogenic role in breast carcinoma.

Downregulation of miR-200 family members, which have been implicated in EMT, correlates with invasiveness and metastasis in various types of tumors including breast, ovarian and renal cancers, suggesting that the miR-200 family regulates a common pathway involved in tumor metastasis. The miR-200 family has five members: miR-200a, miR-200b, miR-200c, miR-141 and miR-429, which are generated from two transcripts. The miR200a/miR-200b/miR-429 cluster is derived from 1p36 and miR-200c/miR-141 is derived from 12p13. The sequences of the members of the miR-200 family are highly similar, especially in the seed region, indicating that they bind to the same targets. Neves et al. [30] demonstrated that the miR-200c/141 cluster is repressed by DNA methylation of a CpG island located in its promoter region. In breast cancer cell lines lacking the expression of miR-200 and miR-141,

the demethylating agent 5-aza-2′-deoxycytidine upregulated the expression of miR-200/miR-141 in a dose-dependent manner, and DNA methylation of the miR-200c/141 promoter correlated with the invasive capacity of human breast cancers. Induction of EMT by the EMT transcription factor TWIST was accompanied by increased DNA methylation and decreased transcription of the miR-200c/141 locus [30].

Promoter methylation of the miR-200 family could be induced by prolonged exposure to autocrine transforming growth factor-β (TGF-β), resulting in transcriptional silencing of miR-200 genes [37]. However, this induction is reversible. TGF-β is a cytokine that suppresses cell proliferation in the early stage of tumorigenesis but promotes cancer cell migration and metastasis in late stage tumor progression, and the latter function is mediated by EMT-inducing transcription factors snail, slug, zinc finger E-box binding homeobox 1 (ZEB1), and ZEB2. miR-200 downregulates ZEB1/2 by direct binding to the 3′-UTRs of ZEB1/2. TGF-β-mediated downregulation of the miR-200 family leads to the upregulation of ZEB1/2 proteins, suggesting a role of the autocrine TGF-β/miR-200/ZEB network during breast carcinoma progression.

Let-7a-3, located on 22q12, is a member of the let-7 miRNA family which regulates the self renewal and tumorigenicity of BT-ICs. The CpG island of the let-7a-3 promoter is also frequently methylated in breast cancer, and its expression is downregulated by the methylation. Furthermore, methylation of let-7a-3 is significantly associated with higher tumor grade and negative ER or PR status but is not associated with patient survival [38].

miR-335, located at 7q32, regulates a number of genes that are associated with increased risk of distal metastasis in different types of human cancers. As a metastasis suppressor miRNA, genomic deletion of miR-335 is a common event in breast cancer metastases. Promoter hypermethylation also leads to the silencing of miR-335 but can be reversed by treatment with demethylating agent [33]. The miR-335 sequence is located in the second intron of the *Mest* gene, and their expressions in breast cancers strongly correlate, suggesting that the primary miR-335 transcript and the *Mest* gene primary transcript are the same, and that the mechanisms regulating the *Mest* gene also dictate miR-335 expression. There are three CpG islands in the promoter region of the *Mest*/miR-335 gene. Analysis of breast cancer cell lines and their metastatic derivatives revealed that methylation of the third CpG island was most strongly correlated with miR-335 silencing, and hypermethylation of this region was also observed in distant metastases from breast cancer patients. miR-335 not only suppresses tumor metastasis but also inhibits tumor re-initiation [33].

The gene for the miR-195/miR-497 cluster is also methylated in breast cancer. Methylation causes downregulation of both miRNAs [31], and with significant downregulation, their expression levels are highly correlated in breast cancer. When their promoter was hypermethylated, expression was increased in cells treated with the demethylating agent 5-aza-CdR [31]. Functionally, re-expression of the two miRNAs in breast cancer cell lines with lower expression levels suppresses cell proliferation and invasion, indicating a tumor suppressor role for these miRNAs in

breast tumor. Raf1 and Ccnd1 have been identified as novel direct targets of miR-195 and miR-497 in different experimental systems [31].

miR-125b is downregulated in several malignancies including breast cancer, and could function as a tumor suppressor that targets multiple oncogenes including ETS1, ERBB2 and ARID3B [28]. Again, the promoter of the miR-125b gene is hypermethylated in breast cancer cell lines and clinical samples, and its expression can be restored by 5-aza-CdR treatment, indicating that promoter methylation is a major mechanism for the downregulation of miR-125b [28].

miR-31 is another miRNA that plays a role in breast cancer progression and metastasis, as its downregulation enhances multiple steps of the invasion-metastasis cascade, including local invasion, extravasation and survival in the circulation system, and metastatic colonization. miR-31 targets several metastatic genes such as RhoA and WAVE3. Like miR-335, miR-31 is also located within the intronic sequence of a non-coding RNA, *LOC554202*, and its transcription shares the same promoter as its host gene [26]. In breast cancer, particularly triple negative breast cancer, promoter hypermethylation is the major mechanism for silencing of the miR-31 gene and its host gene, and treatment of breast cancer cells with either a demethylating agent alone or in combination with a de-acetylating agent significantly increases the transcription of miR-31 and its host gene [26].

miR-203 is a novel regulator for epithelial cell differentiation and has been identified as a putative tumor suppressor gene. The promoter of the miR-203 gene is hypermethylated in several tumors including gastric, hematological, hepatocellular and oral cancers. In our recent study, we found that in breast carcinoma, miR-203 was upregulated in primary clinical tumor samples and some tumorigenic but non-metastatic cell lines but was significantly downregulated in metastatic basal-like cell lines including BT549, Hs578T, and MDA-MB-231 [6]. One major mechanism for the silencing of miR-203 in metastatic breast cancer cells was DNA methylation in its promoter region. Functionally, *in vitro* ectopic expression of miR-203 in BT549 and MDA-MB-231 breast cancer cell lines induced cell cycle arrest and apoptosis. The ability of tumor cell migration and invasion was also inhibited by enforced miR-203 expression. Bioinformatic analysis and biochemical experiments indicate that the snail homolog 2 (SNAI2 or SLUG), a transcription factor enhancing cell invasion and tumor metastasis, was a putative target of miR-203 [6].

6.4.2 DNA Hypomethylation Contributes to miRNA Dysregulation in Breast Cancer

In about two thirds of breast cancers, estrogen receptor alpha (ERα) is upregulated to cause abnormal cellular proliferation. At least one miRNA, miR-375, has been identified as a key mediator of the pro-proliferative function of ERα [34]. In ERα-positive breast cancer cells, miR-375 is upregulated by loss of epigenetic marks including H3K9me2 and DNA hypomethylation, which results in the dissociation

of the CTCF transcription repressor from and the association of ERα to the promoter of miR-375 [34], although one of the two CpG islands is still hypermethylated in breast cancer cells. A direct target of miR-375 miRNA, RASD1, was identified, which negatively regulates ERα expression, suggesting a positive regulatory feedback loop between ERα and miR-375 in breast cancer cells.

6.4.3 DNA Methylation Regulates miRNA Biogenesis-Related Genes in Breast Cancer

In addition to miRNAs themselves, the genes responsible for the biogenesis and transportation of miRNAs can be also regulated by DNA methylation, which then influences the expression of a set of miRNAs in breast cancer. Expotin-5 (XPO5), a protein responsible for exporting pre-miRNAs from the nuclei to cytoplasm, is overexpressed in breast tumors and is regulated by both genetic and epigenetic mechanisms. At the genetic level, two missense SNPs located in the coding region of XPO5 are significantly associated with breast cancer risk in post-menopausal women [13]. Decreased methylation at the XPO5 promoter was associated with both XPO5 expression and increased breast cancer risk. Functional analyses of mutant XPO5 in cells from different types of cancers showed that genetic defects in the XPO5 gene cause trapping of pre-miRNAs in the nucleus, reduce miRNA transportation and processing, and diminish miRNA-mediated inhibition of oncogenes such as *EZH2* (inhibited by miR-26a), *MYC* (inhibited by miR-192, miR-215, let-7, and miR-24), *K-RAS* (inhibited by miR-192, miR-215, and let-7) and ZEB1 (inhibited by the miR-200 family) [39]. When wild-type *XPO5* was ectopically expressed, miR-200 transcript was upregulated, downregulating *ZEB1* and its targets E-cadherin, *CRB3*, *INADL*, and *LGL2*. These findings suggest that XPO5 plays a tumor suppressive role in breast tumorigenesis by regulating the processing and expression of multiple miRNAs.

RBM38, an RNA-binding protein (RBP) controlling the access of miRNAs to their target mRNAs, is induced by p53 to modulate the action of p53-controlled miRNAs, which is necessary for proper function of p53. RBM38 is required for decreased miRNA accessibility to a number of p53-induced transcripts, allowing an optimal target gene induction and cell cycle control. Analysis of the methylation status of the RBM38 promoter in 58 clinical breast cancer samples with wild-type p53 and 44 with mutated p53 demonstrated that the RBM38 promoter is methylated in 26 % of samples with wildtype p53 and in 7 % of samples with mutant p53. DNA hypermethylation of RBM38 is significantly associated with p53 mutation status, and RMB38 expression is indeed reduced in the samples with methylated RMB38 promoter [40]. Furthermore, treatment of two breast cancer cell lines with methylation in the RBM38 promoter with 5-aza-CdR induced RBM38 expression. These findings indicate a novel layer of p53 regulation, and RBM38 seems to be necessary for the tumor-suppressive function of p53.

6.5 Histone Modification Regulates miRNA Expression in Breast Cancer

Histone modification is another important regulatory mechanism that modulates gene transcription, and aberrant changes in histone modification in breast cancer correlate with tumor phenotype, prognostic factors, and patient outcome. Histone modification can be affected by DNA methylation and vice versa, and changes in histone modifications also modulate miRNA expression in breast carcinoma (Figs. 6.1 and 6.2; Table 6.2).

As mentioned above, promoter methylation downregulates miR-125b, which upregulates two targets of miRNA, HER2 and ER. Gain of histone methylations H3K9me3 and H3K27me also result in the silencing of miR-125b [47]. It has been shown that in normal breast tissues, the transcription regulator CCCTC-binding factor (CTCF) binds to the promoter of the miR-125b locus to prevent DNA methylation and the formation of repressive histone marks H3K9me3 and H3K27me, resulting in an open chromatin conformation for the transcription of miR-125b. In breast cancer cells, however, loss of CTCF leads to hypermethylation of miR-125b promoter and the gain of histone repressive marks, which blocks the transcription of miR-125b. Similar alterations have also been observed in ovarian and cervical cancers (Fig. 6.3).

miR-375 is another miRNA that is repressed by histone methylation H3K9me2 and promoter DNA [34]. Although the transcriptional repressor CTCF is also involved in the regulation of miR-375, it appears that hypomethylation of the miR-375 promoter region is necessary for CTCF's binding and subsequent recruitment of histone methyltransferases such as ZEB1 to the promoter of miR-375, which is in contrast to the regulation of miR-125b by CTCF. Consistently, the repressive mark H3K9me3 on the miR-375 promoter is associated with the repression of miR-375 expression [34]. These epigenetic alterations also influence the binding of ERα to the regulatory regions of miR-375 (Fig. 6.3).

Table 6.2 List of miRNAs that are dysregulated by alterations in histone methylation and acetylation in breast cancer

miRNA	Target(s) identified	Functional notes	References
let-7e[a]	Cyclin D1	Arrests the cell cycle	[41]
miR-27a[b]	Foxo1	Promotes tumorigenesis	[42]
miR-125b[a]	STARD13, ARID3B, ETS1, Mucin1, Bak1, C-Raf, Her2, ESR1	Induces proliferation and metastasis. Predicts chemoresistance	[43]
miR-126[b]	VEGFA, PIK3R2, IRS-1	Suppresses tumor growth and metastasis	[44]
miR-155[b]	RhoA, FOXO3A, SOCS1	Inhibits cell survival, growth, EMT, and chemoresistance	[45]
miR-200a[b]	Keap1, SIRT1	Regulates EMT	[46]
miR-320[b]	—		[49]
miR-375[a]	RASD1	Drives proliferation of ER+ cells	[34]

[a]Regulated by histone methylation
[b]Regulated by histone acetylation

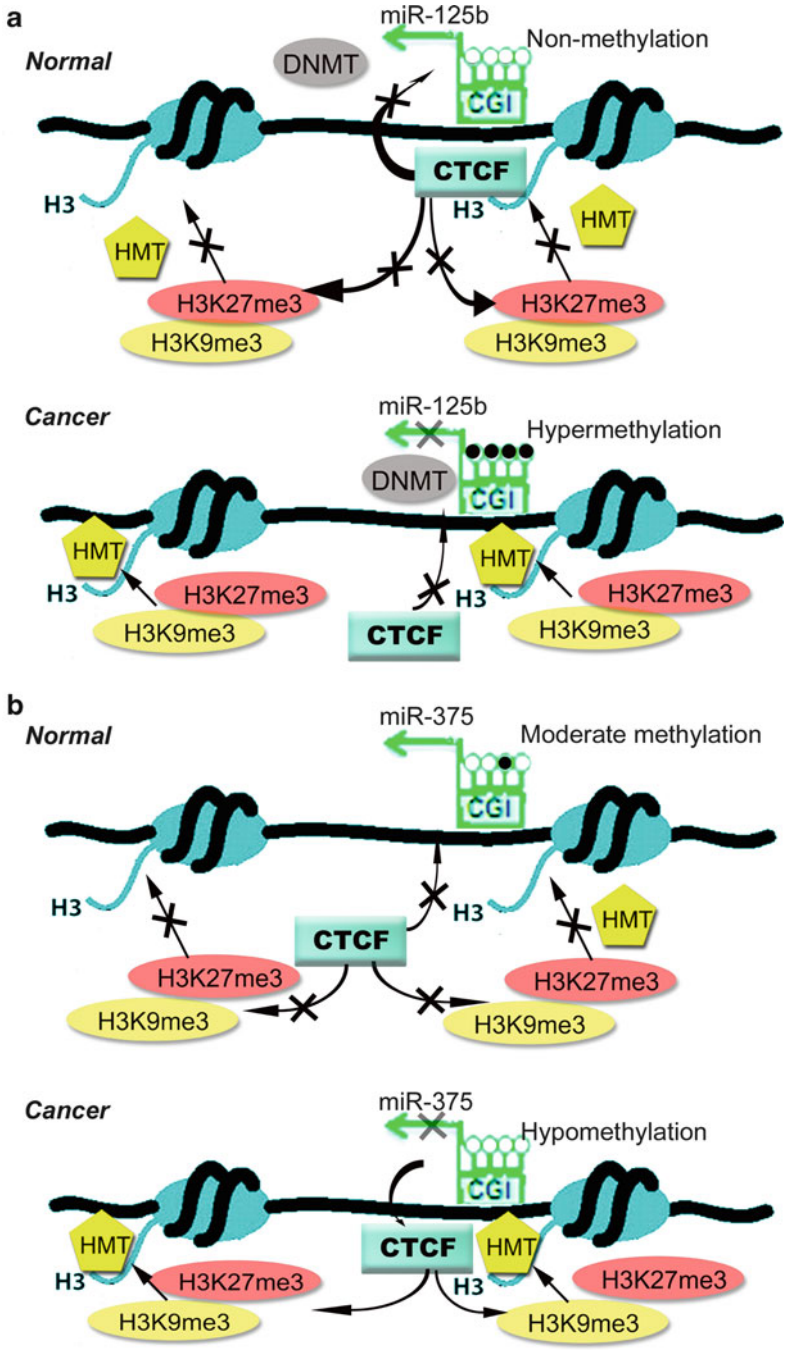

Fig. 6.3 Models of the mechanisms by which miR-125b (**a**) and miR-375 (**b**) are silenced by epigenetic mechanisms involving the transcriptional factor (*CTCF*) in cancer cells**.** Although both miR-125b and miR-375 are regulated by DNA methylation, histone modification, and CTCF, the molecular mechanisms are different as discussed in the text

The nuclear protein JARID1B, which belongs to the ARID family of DNA binding proteins and has strong transcriptional repressive activity, is upregulated in breast cancer with the demethylation of trimethylated lysine 4 on histone 3 (H3K4me3), which is an active chromatin mark [41]. Knocked down JARID1B expression in MCF-7 breast cancer cells caused expression changes in multiple miRNAs, including the downregulation of the let-7c tumor suppressive miRNA [41]. JARID1B binds to the promoter of let-7e to remove the H3K4me3 histone mark, leading to active expression of let-7c and the downregulation of cyclin D1, a direct target of let-7e. Knockdown of JARID1B downregulates let-7c and regulates cyclin D1 as predicted, which results in an accumulation of MCF-7 cells in the G1 phase. Therefore, the JARID1B demethylase contributes to tumorigenesis at least via the epigenetic suppression of tumor suppressive miRNAs.

BRCA1, a well established tumor suppressor gene, is frequently mutated in familial breast and ovarian cancers. BRCA1 functions in multiple cellular pathways such as DNA damage and repair, protein ubiquitination, chromatin remodeling, and transcriptional regulation. R1699Q, a point mutation in the C-terminal BRCT domain of BRCA1 that is associated with increased predisposition to breast cancer, does not impair DNA damage and repair but upregulates the oncogenic miRNA miR-155, as demonstrated in mouse embryonic cells [45]. Further studies have demonstrated that BRCA1 represses the expression of miR-155 via its interaction with the HDAC2 complex, which deacetylates histones H2A and H3 on the miR-155 promoter [45].

Epigenetic aberrations can also cause resistance to chemotherapy in breast cancer. Several HDAC inhibitors and demethylation agents have been used to treat drug-resistant breast cancers, and these treatments can cause rapid changes in mRNA expression [48]. For example, the HDAC inhibitor LAQ824 upregulated five miRNAs while downregulating 22 miRNAs in the SK-BR-3 breast cancer cell line [48]. In addition, during the suppression of clonogenicity of apoptosis-resistant MCF-7TN-R breast cancer cells mediated by HDAC inhibitor trichostatin A (TSA), 22 miRNAs were upregulated and 10 downregulated [49]. Consistently, treatment with the HDAC inhibitor SAHA in breast cancer cells results in the re-expression of miR-200a and the downregulation of its target gene Keap1 both *in vitro* and *in vivo* [50]; inhibitors of DNA methylation and HDAC upregulate miR-126 and its host gene EGFL7 in breast cancer cells [44]. These findings indicate the importance of post-transcriptional regulation by histone modifying enzymes in breast cancer formation, progression and chemotherapy resistance.

Early exposure to xenoestrogens may increase the risk of breast cancer in adult life, and epigenetic regulation of multiple miRNAs appears to be involved. In epithelial cells pre-exposed to diethylstilbestrol, a large number of miRNAs were altered. Further characterization of a downregulated miRNA, miR-9-3, demonstrated that two repressive chromatin marks, H3K27me3 and H3K9me2, occurred at the promoter region of miR-9-3 [25], which was accompanied by the recruitment of DNMT1 to the promoter to cause DNA hypermethylation and transcriptional silencing [25].

6.6 miRNAs Also Regulate Epigenetic Events in Breast Tumorigenesis

Cancer-associated miRNAs also alter the epigenetic landscape by way of DNA methylation and histone modification. EZH2, a histone methyltransferase contributing to the epigenetic silencing of target genes and thus regulating the development and progression of breast cancer, is overexpressed in aggressive tumors, and several miRNAs have been reported to target EZH2 in tumorigenesis. miR-101 is one such miRNA whose genomic deletion frequently occurs in cancers, leading to the upregulation of EZH2 in cancer cells [4]. Another miRNA, miR-26a, suppresses apoptosis to facilitate carcinogenesis by targeting MDTH and EZH2 in breast cancer [51]. Furthermore, silencing miR-214 in breast cancer cells increases cell proliferation, invasion, and accumulation of the EZH2 methyltransferase [52]. On the other hand, stable overexpression of miRNAs such as miR-7 and miR-218 could reactivate tumor suppressor genes such as RASSF1A and claudin-6 by modulating DNA methylation and histone modification [53]. miR-10a inhibits HoxD4 expression at the transcriptional level by targeting a homologous DNA region in the promoter region of the *hoxd4* gene, and reduced HoxD4 expression was accomplished by *de novo* DNA methylation and trimethylation of histone 3 lysine 27 (H3K27me3) at the promoter of HoxD4 [54]. SIRT1, a member of the class III HDAC inhibitors, is regulated by miR-200a during EMT in mammary epithelial cells [46].

6.7 Conclusions and Future Perspectives

During the last several years, an increasing number of studies have been published establishing the role of DNA methylation and histone modification in the transcriptional regulation of miRNAs and their dysregulation during the development and progression of human cancers. Studies are also emerging that indicate a role of miRNAs in the regulation of epigenetic modifications such as DNA methylation and histone modification. In addition to the identification of novel miRNAs that are regulated by DNA methylation and histone modification, it is of even greater interest to understand the functions and mechanisms of miRNAs in the regulation of histone modification and DNA methylation as well as the abnormalities of these mechanisms in human cancers. In addition to epigenetic modifications, other epigenetic mechanisms including higher-order chromosome structures could also modulate miRNA expression in breast carcinomas.

Significant progress has been made in the discovery and evaluation of small molecules and compounds that inhibit histone-modifying enzymes such as HDACs in the treatment of cancer. Considering that miRNAs are small polynucleotides, the development of miRNA-based drugs to target the epigenetic programs of breast cancer cells is plausible, which could provide effective therapies for breast cancer with higher specificity and longer lasting effects.

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Chapter 7
Epigenetic Variations of Stem Cell Markers in Cancer

Sripathi M. Sureban, Dongfeng Qu, and Courtney W. Houchen

Abstract Epigenetic mechanisms play an important role in the regulation of gene expression and are critical for the function of normal physiological processes like cell proliferation, differentiation and morphogenesis. Alterations in epigenetic mechanisms contribute to the initiation and progression of various pathological conditions like genetic disorders, autoimmune diseases, aging and cancer. In this chapter, we discuss the different types of epigenetic mechanisms and how dysregulation of these mechanisms can lead to the initiation and development of various cancers. In addition, we highlight the importance of cancer stem cell (CSC) markers that serve as putative biomarkers for cancer diagnosis and prognosis, and epigenetic variations of these markers in cancer. Given the importance of microRNAs in gene regulation and cancer, we discuss the regulation of various tumor suppressor microRNAs by CSC markers and vice versa. A thorough knowledge on these aspects is critical in the development of therapeutics that can target cancer stem cells.

S.M. Sureban • C.W. Houchen (✉)
Department of Medicine, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

Department of Veterans Affairs Medical Center, Oklahoma City, OK 73104, USA

Peggy and Charles Stephenson Oklahoma Cancer Center, Oklahoma City, OK 73104, USA
e-mail: courtney-houchen@ouhsc.edu

D. Qu
Department of Medicine, The University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

Department of Veterans Affairs Medical Center, Oklahoma City, OK 73104, USA

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7.1 Introduction

Cancer is a complex disease with many different causes, which involves both genetic and epigenetic alterations. It can be caused by genetic mutations, down-regulation of tumor suppressors, activation of oncogenes, and epigenetic alterations. There is increasing evidence to support the existence of cancer stem cells (CSCs) or cancer initiation cells. This population of cells possesses similar characteristics of adult tissue stem cells, including the abilities to self-renew, differentiate, activate telomerase expression, activate anti-apoptotic pathways, and migrate and metastasize. In this chapter, we will focus on the epigenetic variations of cancer stem cell markers.

Accumulation of mutations in multiple critical genes is believed to cause the transformation of a normal mucosal epithelial cell into a cancer cell [1]. Mutations in *APC*, *KRAS*, *TP53*, MMR family genes, *CTNNB1*, and *SMAD4* have been implicated in colon tumorigenesis, for example. In cancer, the CpG island methylator phenotype (CIMP) is characterized by hypermethylation of the CpG island in the promoter regions of many tumor suppressor and DNA repair genes, resulting in inactivation of these genes [2]. Other epigenetic alterations in cancer include DNA methylation, histone modification, microRNA levels, microsatellite instability, and chromosomal instability [3].

7.2 Epigenetic Variations

Epigenetic alterations play an important role in the development and progression of various cancers. The disruption of epigenetic processes often leads to altered gene function and result in malignant cellular transformation. The cancer epigenome is characterized by global changes in DNA methylation, modifications in histone proteins and alterations in the expression of chromatin-modifying enzymes [4]. The epigenetic changes can affect the expression profiles of various genes and therefore contribute to the initiation and progression of various pathological conditions. Some epigenetic alterations can either lead to inactivation of tumor suppressor genes or activation of oncogenes, which can ultimately result in cancer. According to the 'two-hit' hypothesis of carcinogenesis proposed by Knudson [5], loss of function of both alleles in a gene (e.g. tumor suppressor gene) is needed for malignant transformation. The first hit may occur in the form of a mutation in the gene, followed by loss of the wild-type allele either through deletion or loss of heterozygosity. Ultimately, inactivation of the regulatory genes can lead to the development of cancer [6].

7.2.1 Aberrations in DNA Methylation and Cancer

Many human tumors display genome-wide hypomethylation and hypermethylation. Thus, the changes in DNA methylation can lead to the initiation and progression of cancer. DNA hypomethylation can occur at many genomic sequences including repetitive elements, CpG poor promoters, retrotransposons, introns and gene deserts [7]. DNA hypomethylation at repeat sequences promotes chromosomal rearrangements and can result in increased genomic instability. Hypomethylation of DNA can also activate oncogenes. The induction of genomic instability by hypomethylation is best exemplified in patients with the immunodeficiency, centromeric region instability and facial anomalies syndrome, in which a germ line mutation in the DNMT3B gene results in hypomethylation and subsequent genomic instability [4].

Hypermethylation of many tumor suppressor genes such as p53, Rb, p16, BRCA1, SFRP and WIF1 has been reported in various tumors. Epigenetic silencing of such tumor suppressor genes by hypermethylation can lead to tumor initiation and serve as the second hit in the Knudson's 'two-hit' hypothesis of carcinogenesis. In addition to direct inactivation of tumor suppressor genes, hypermethylation of DNA can also indirectly silence DNA repair genes (e.g. MLH1, BRCA1) and transcription factors (e.g. GATA4, GATA5, RUNX3) in different tumors [4, 8, 9].

7.2.2 Histone Modifications and Cancer

Histone modifications including acetylation, methylation, phosphorylation, and ubiquitination play an important role in structural changes of chromatin. Histone acetylation is a global mark of gene activity. While histone deacetylases (HDACs) remove histones, histone acetyl transferases (HATs) acetylate histones. Changes in histone acetylation can also contribute to the initiation and progression of tumors. HDACs are often found overexpressed in various types of cancer. Loss of histone acetylation can result in gene repression. Cancer cells display widespread changes in histone methylation patterns in addition to changes in histone acetylation. Nguyen et al. [10] demonstrated that aberrantly silenced genes in cancer cells exhibit a heterochromatic structure that is characterized by histone H3 lysine 9 (H3-K9) hypermethylation and histone H3 lysine 4 (H3-K4) hypomethylation. They suggest that H3-K9 methylation might play a critical role in silencing the tumor-suppressor genes in cancer.

7.2.3 Nucleosome Modification

Nucleosome remodeling works in concert with DNA methylation and histone modifications and therefore plays a central role in tumor-specific gene silencing. Nucleosome remodeling can lead to aberrant gene silencing through the transmission of repressive epigenetic marks to tumor suppressor gene promoters [4]. The

SWI/SNF family of chromatin remodeling complexes regulates the action of transcription factors and targeted gene expression. SWI/SNF complexes are involved in either enhancement or suppression of the downstream genes. Alterations in the SWI/SNF complex are also associated with cancer development. These complexes have a widespread role in tumor suppression, as inactivating mutations in several SWI/SNF subunits have been identified at high frequency in a variety of cancers [4].

7.2.4 Histone Variants

The variants of the histone H2A family are the most diverse in sequence and exhibit distinct functions, comprising DNA damage repair, transcriptional regulation, cell cycle control and chromatin condensation [11]. The histone variant H2A.Z has been implicated in various biological processes including gene regulation and genomic stability. Deregulation of the histone variant H2A.Z has been implicated in the development of several tumors. H2A.Z is overexpressed in several types of cancer and has been associated with the promotion of cell cycle progression. Loss of H2A.Z is also implicated in tumor progression through destabilization of chromosomal boundaries resulting in the spread of repressive chromatin domains and *de novo* hypermethylation of tumor suppressor gene promoters [4, 12]. Loss of another H2A variant, MacroH2A1.1, is associated with lung and colorectal cancer, and probably is a general feature of tumorigenesis [13].

7.2.5 MicroRNAs

MicroRNAs (miRNAs) are a class of highly conserved small non-coding RNA molecules (21–25 nucleotides) that bind to target mRNA. Upon binding, target mRNA is either degraded or translationally repressed. miRNAs can act as oncogenes or tumor suppressors. miRNAs can regulate the expression of hundreds of target mRNAs simultaneously and therefore control a variety of cell functions including cell proliferation, self-renewal, stem cell maintenance and differentiation. miRNAs can induce aberrant DNA methylation in hepatocellular carcinoma, lung cancer, and acute myeloid leukemia by directly targeting DNA methyltransferases [14].

7.3 Stem Cells and Cancer

Stem cells are an essential component of embryonic development, tissue homeostasis, mucosal renewal following injury and the development of neoplasia. Adult stem cells have the unique capacity for self-renewal under basal conditions and during tissue repair. Identification of specific stem cell markers in hematopoietic

tissue has revolutionized the study of stem cell biology and has paved the way for bone marrow transplantation as a viable therapy for many liquid and solid tumors. Potential tumor stem cells have been recently identified from breast [15] and other tissues [16–19]. Identification of similar stem cells or tumor stem cells in the gastrointestinal tract has been more elusive. There have been several candidate markers of gastrointestinal stem cells including LGR5 [20], BMI1 [21], HOPX [22], DCLK1 (formerly known as DCAMKL-1) [23], and Musashi-1 (MSI-1) [24, 25]. Normal stem cells and cancer cells have certain features in common. These include the ability to (*a*) self-renew, (*b*) differentiate, (*c*) activate telomerase expression, (*d*) activate anti-apoptotic pathways, and (*e*) migrate and metastasize [26]. In addition to these properties, anchorage independent growth has been a common feature of transformed cells and normal stem cells [27–30]. Impaired stem cell self-renewal is also a critical early event in neoplastic transformation. Under normal conditions, a stem cell is able to produce an exact copy of itself as well as a daughter cell that undergoes differentiation into the lineages found in differentiated tissues [26]. During normal development, stem cell self-renewal is regulated by signals from the surrounding stem cell "niche". Deregulation of this self-renewal process leading to stem cell expansion, may be a key early event in carcinogenesis. Recently, critical pathways that regulate the self-renewal of normal stem cells including Wnt, Notch, and Hedgehog, have begun to be elucidated. Defects in the Wnt signaling pathway are seen early in colon cancer carcinogenesis and more recently, in human pancreatic, gastric, prostate, and breast carcinomas [31, 32].

7.4 Cancer Stem Cell Markers

The existence of CSCs was first demonstrated in acute myeloid leukemia [16] and subsequently verified in breast [15], pancreatic [17] and brain tumors [18, 19]. $CD133^+$ subpopulations isolated by fluorescent activated cell sorting (FACS) from brain tumors can initiate clonally derived neurospheres *in vitro* that showed self-renewal, differentiation, and proliferative characteristics similar to normal brain stem cells [18, 19]. Furthermore, transplantation of $CD133^+$, but not $CD133^-$ cells into NOD/SCID mice was sufficient to induce tumor growth *in vivo*. These cells have been termed CSCs because, like normal stem cells, they can both self-renew and produce differentiated progeny. Recently, primary human pancreatic adenocarcinomas were implanted in immunocompromised mice to assess the utility of specific cell surface markers to identify a subpopulation of pancreatic cancer cells with enhanced tumorigenic potential [17]. A subpopulation of $CD44^+CD24^+$ and epithelial surface antigen $(ESA)^+$ cells was identified as putative pancreatic cancer stem cells [17]. This was the first study to identify a subpopulation of tumor initiating stem cells in the pancreas. In this book chapter, we will focus on epigenetically regulated, putative CSC markers like BMI1, CD133, CD44, DCLK1, MSI-1 and ALDH1.

7.4.1 Cancer Stem Cell Markers as Putative Biomarkers for Cancer Diagnosis and Prognosis

Identifying biomarkers is essential for cancer prevention, screening, intervention, and surveillance. In general, cancer biomarkers can be divided into three categories: biomarkers for risk factors, biomarkers for early detection, and biomarkers for disease prognosis. Several intestinal and colon stem cell markers such as *DCLK1*, *LGR5*, *BMI1*, and *HOPX* are implicated as potential prognostic markers in colorectal cancer [33–36]. These putative biomarkers were identified in biopsy tissue samples or plasma samples of colorectal cancer patients. Hypomethylation of CD44, CD133, and MSI-1 has been suggested as a biomarker for triple negative breast cancer (TNBC) [37].

7.4.2 Methylation of Cancer Stem Cell Markers

7.4.2.1 BMI1

BMI1 is a member of the polycomb group genes (PcG) that are highly conserved throughout evolution. PcG proteins are epigenetic regulators and they are related to three types of epigenetic processes. These include DNA methylation, histone modification and non-coding RNA regulation. BMI1 is a part of polycomb repressive complex-1 (PRC-1) of the RING-finger domain that transcriptionally represses genes subsequent to histone H3 27 methylation in order to maintain repression. BMI1 in PRC-1 complex is required to stimulate the E3 ubiquitin-protein ligase activity of RNF2/RING2, resulting in ubiquitination of histone H2A [38]. Lineage tracing studies indicate that BMI1 is an intestinal stem cell (ISC) marker, labeling the quiescent stem cell populations [21]. It is overexpressed in different cancer types [38]. BMI1 upregulation is associated with malignant transformation and acquisition of the malignant phenotype in liver cancer (HCC) [39]. A recent study has demonstrated that inhibition of DNA methyltransferase (DNMT), an enzyme that catalyzes the transfer of a methyl group to DNA and regulates gene expression, by 5-AzaC treatment results in decreased expression of BMI1. This indicates that 5-AzaC treatment has an effect on BMI1 itself or on negative mediators of BMI1. Considering BMI1 expression is regulated at the mRNA level, the mediators, if any, would regulate the BMI1 mRNA. One such group of mediators that regulate BMI1 mRNA is miRNAs. *miR-200c* is underexpressed in various cancers and is known to suppress BMI1 mRNA. Indeed following inhibition of DNMT, an increased expression of *miR-200c* was observed resulting in the decreased expression of BMI1. Histone-lysine N-methyltransferase (EZH2), another member of PcG family, is also downregulated by DNMT inhibition similar to BMI1. Inhibition of BMI1 resulted in cellular senescence [40]. Additionally, BMI1 acts as an oncogene and along with c-Myc is responsible for initiation of lymphoma [38]. These studies demonstrate that BMI1 is an epigenetic regulator of tumor suppressor genes and BMI1 itself can be epigenetically regulated by miRNAs.

7.4.2.2 DCLK1

Doublecortin-like kinase 1 (DCLK1), also known as DCAMKL1, is a microtubule associated protein kinase that is expressed in post-mitotic neurons. It controls neurogenesis by regulating mitotic spindles and M phase progression. It is required for proper neuronal migration and axonal wiring. It is a putative neural progenitor cell marker [36]. Several reports have suggested that DCLK1 is a putative ISC marker. DCLK1 is overexpressed in several solid tumors, including colon, pancreatic, liver, breast, lung, and renal cancers [41]. Introducing the DCLK1 specific siRNA into colon cancer cell lines down-regulated several oncogenes expression and decreased cell proliferation. Furthermore, introducing the DCLK1 specific siRNA into solid tumor xenografts originated from human colon cancer and pancreatic cancer cell lines down-regulated several oncogenes expression levels and also inhibited tumor growth [41, 42]. These data suggest that DCLK1 is also a putative cancer stem cell marker. Recent report by Nakanishi et al. clearly indicates that DCLK1 positive cells are the tumor stem cells in intestinal tumor development [43]. Andresen et al. studied the methylation of DCLK1 in cholangiocarcinoma patients, and found that high methylation frequency of DCLK1 is related to the tumor, and unmethylated DCLK1 in control samples [44]. Furthermore, in that study, several cancer cell lines also had significant DCLK1 promoter methylation. These data taken together indicate that DCLK1 promoter methylation can be considered as potential biomarker for cholangiocarcinoma detection [44]. Though this report suggests that DCLK1 promoter is hypermethylated in tumor, other studies have demonstrated that DCLK1 is upregulated in various human cancers and can regulate various tumor suppressor miRNAs. siRNA-mediated knockdown of DCLK1 resulted in: increased expression of miRNA *let-7a,* leading to downregulation of oncogenes like c-Myc and KRAS; increased expression of *miR-144* miRNA and a corresponding decrease in Notch1 and finally increased expression of *miR-200a* leading to downregulation of epithelial-mesenchymal transition (EMT) transcription factors like ZEB1, ZEB2, Snail, and Slug in human colorectal and pancreatic cancer cell lines and tumor xenografts (Fig. 7.1). Recently, we have observed that the knockdown of DCLK1 resulted in increased expression of *miR-143/145* cluster leading to decreased expression of pluripotency factors like OCT4, SOX2, KLF4 and Nanog [41, 42, 45]. These data taken together indicate that DCLK1 may regulate tumor suppressor miRNAs in an epigenetic manner.

7.4.2.3 Musashi-1 (MSI-1)

MSI-1 is a RNA-binding protein that regulates the translation of its target mRNA including mNumb, and other genes involved in cell cycle regulation, proliferation, and apoptosis. It was originally reported as a neural tumor cell marker. Recent studies also indicated that MSI-1 is an ISC marker. Overexpression of MSI-1 was found in various tumors including colon, brain, breast, medulloblastoma, glioma, gastric and cervix [46]. Introducing the MSI-1 specific siRNA into colon cancer cell lines down-regulated expression of several oncogenes and decreased cell proliferation.

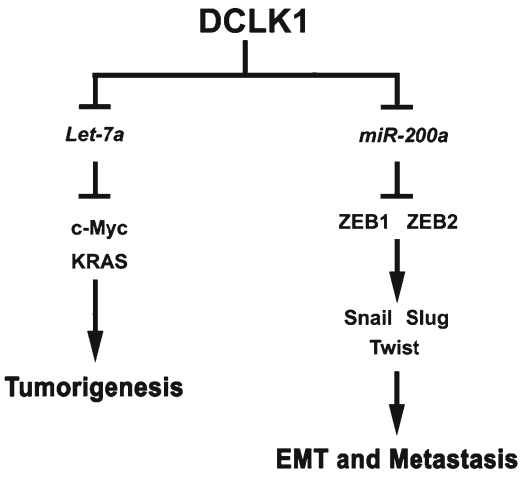

Fig. 7.1 DCLK1 inhibits tumor suppressor miRNAs *let-7a* and *miR-200a*. DCLK1 positively regulates key oncogenic signaling pathways like c-Myc and KRAS by inhibiting miRNA *let-7a.* DCLK1 also positively regulates ZEB1, ZEB2, Snail and Slug, and induce EMT and metastasis by inhibiting *miR-200a*

Furthermore, introducing the MSI-1 specific siRNA into solid tumor xenografts generated using human colon cancer cell lines inhibited tumor growth via induction of mitotic catastrophe. Tumors treated with specific MSI-1 siRNA demonstrated a significant downregulation of Notch1 and upregulation of p21 [25]. Overexpression of MSI-1 in breast epithelial cells resulted in activation of CSC signaling pathways Notch1 and WNT [46]. A study by Kagara et al. found that hypomethylation of MSI-1 promoter region is related with TNBC [37]. Epigenetic regulation governing the hypomethylation of MSI-1 promoter is responsible for MSI-1 gene activation and subsequent progression of breast cancer [37]. Another study by Vo et al. demonstrated that MSI-1 is regulated by various tumor suppressor miRNAs like *miR-34a, miR-101, miR-128, miR-137* and *miR-138*. The authors suggest that these miRNAs are direct regulators of MSI-1 expression during tumorigenesis of malignant nervous system tumors [47]. We have demonstrated that MSI-1 regulates tumor suppressor miRNAs *let-7a* and *miR-200a* in colorectal cancer cells. siRNA-mediated knockdown of MSI-1 resulted in increased expression of *let-7a* and *miR-200a* [48]. These data taken together demonstrate the existence of epigenetic variations of MSI-1 and highlights that MSI-1 plays an important role in the regulation of miRNAs.

7.4.2.4 CD44

CD44 is a polymorphic integral membrane glycoprotein with an extracellular domain, plays important roles in lymphocyte homing, cell-cell adhesion, and cytoskeletal interactions with the extracellular matrix. CD44 is a putative CSC marker for breast, pancreatic, and prostate cancer. Al-Hajj et al. were the first to demonstrate

that $CD44^{+}/CD24^{-/low}$ has stem cell-like properties [15]. p53 inactivation in breast cancer cells (basal-like breast cancer cells, which has stem cell-like properties with high expression of CD44) leads to hypomethylation of IL-6 and a subsequent hypomethylation of CD44 and CD133 which enhances their expression. Additionally, hypomethylation of the CpG island of CD44 promoter region is associated with prostate cancer, TNBC, Hodgkin and non-Hodgkin lymphoma [37]. CD44 methylation is a prognostic marker for prostate cancer. In TNBC samples and several breast cancer cell lines, CD24 was found to be unmethylated. Additionally, increased expression of CD44 has been demonstrated in human TNBC samples and can be used as a prognostic marker [37]. These studies indicate that CD44 undergoes hypomethylation and is increased in various cancers.

7.4.2.5 CD133

CD133, also known as Prominin-1 or AC133, is a cellular surface glycoprotein and putative CSC marker for human breast, liver, prostate, pancreatic, gastric, head and neck, lung and colon cancer. Furthermore, CD133 is also a putative ISC and neural stem cell (NSC) marker. $CD133^{+}$ tumor cells can initiate neurospheres, which exhibit self-renewal, differentiation, and proliferation resembling that of normal NSCs. The $CD133^{+}$ cancer cells have high tumor-initiating capacity. Similar to CD44, hypomethylation of CD133 promoter region has been found in TNBC. Increased expression of CD133 was found in human TNBC samples [37]. Promoter hypomethylation in the CpG islands of CD133 resulted in aberrant expression of CD133 in human gliomas. In colon cancer and glioblastoma culture cells, the promoter CpG island of CD133 is methylated in the cells with low expression of CD133 and hypomethylated in $CD133^{+}$ cells. This epigenetic variation in CD133 gene is due to differential histone modification within the culture cells [49]. These data taken together indicate that CD133 is epigenetically modified in various human cancers.

7.4.2.6 ALDH1

ALDH (Aldehyde dehydrogenase) genes particularly ALDH1 is a hematopoetic stem cell marker. ALDH activity in normal and CSCs converts retinol to retinoic acid leading to cell differentiation. ALDH activity has been characterized in CSCs from human head and neck, colon, breast, liver, and lung tumors. ALDH activity has also been detected in acute myeloid leukemia stem cells. $ALDH^{+}$ breast cancer cells had capability of forming spheroids and tumors compared to $ALDH^{-}$ cells [50]. In one study, ALDH1 was found to be hypomethylated following expression of hepatitis B virus-encoded X (HBx) protein in HepG2, human hepatoma cell line [51]. Another study by Park et al. have shown that $ALDH1^{+}$ breast tumors were less methylated compared to $ALDH1^{-}$ tumors [52].

7.5 MicroRNAs as Regulators of Cancer Stem Cells

Several miRNAs have been shown to regulate CSCs. $CD44^{+}/CD24^{-/low}$ enriched breast cancer cells express low levels of *let-7* and *miR-200*. These miRNAs are markedly reduced in normal stem cells too. Prostate CSCs sorted by $CD44^{+}/CD24^{-/low}$ and CD133 also have significantly low levels of *miR-34a* and *let-7*.

7.5.1 miR-200

miR-200 family of miRNAs is known to inhibit EMT and metastasis. Metastatic breast cancer specimens demonstrated a significant downregulation of *miR-200* compared to non-metastatic tumors. *miR-200* family of miRNAs is downregulated in breast cancer cells which underwent TGF-β-induced EMT. On the other hand, induction of EMT in human mammary epithelial cells results in acquisition of stem-cell like properties ($CD44^{+}/CD24^{-/low}$). Ectopic overexpression of *miR-200* in these cells results in inhibition of EMT via EMT transcription factors ZEB1 and ZEB2 (Fig. 7.2) [50, 53]. These data indicate that downregulation of these miRNAs is an essential step towards EMT and metastasis and these miRNAs play an important regulatory role in CSCs.

7.5.2 let-7 miRNAs

Similar to *miR-200*, *let-7* family members are downregulated in various CSCs and act as tumor suppressors. Overexpression of *let-7a* resulted in inhibition of $CD44^{+}/CD24^{-/low}$ cell proliferation, self-renewal, spheroid formation, tumor growth and

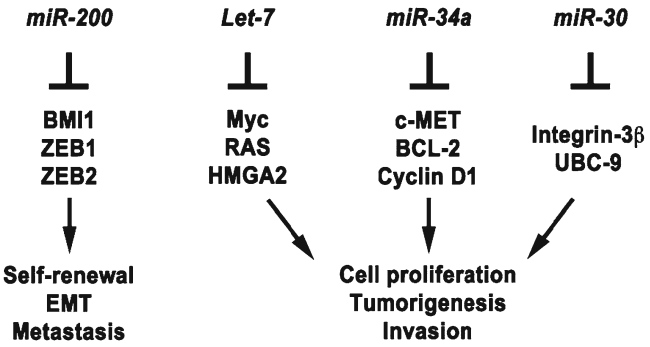

Fig. 7.2 Role of CSC specific miRNAs. *miR-200* family negatively regulates BMI1, ZEB1 and ZEB2 and inhibits self-renewal, EMT and metastasis of CSCs. *Let-7* family negatively regulates Myc, RAS and HMGA2; *miR-34a* inhibits c-MET, BCL-2 and Cyclin D1; *miR-30* inhibits Integrin β3 and ubiquitin-conjugating enzyme 9 (*UBC-9*). The three miRNAs inhibit CSCs proliferation, tumorigenesis and invasion

metastasis in immunocompromised mice. *let-7* regulates various oncogenic pathways like RAS, high mobility group (HMG), and Myc (Fig. 7.2). CSCs that lack *let-7* demonstrate increased expression of HMGA2 and are maintained in undifferentiated state. *let-7* also targets KRAS and c-Myc and possess tumor suppressor properties. In addition, Lin28B and homolog of Lin28 have been shown to regulate *let-7* expression, and Lin28 is a target of *let-7*. These studies demonstrate that *let-7* regulates various critical stem cell signaling pathways that are highly relevant for initiation and progression of cancer [53].

7.5.3 miR-34a

miR-34a was found to be downregulated in glioblastoma CSCs and overexpression of *miR-34a* in these cells resulted in apoptosis, cell cycle arrest and tumor growth arrest via downregulation of critical oncogenes like Notch1, CDK6 and c-MET (Fig. 7.2). In prostate CSCs ($CD44^+$ and $CD133^+$) (human tumors and cell lines), *miR-34a* and *let-7* were underexpressed. Lentiviral-based overexpression of *miR-34a* in prostate CSCs resulted in inhibition of tumor growth and metastasis. A decreased expression of CD44 was also observed. Cyclin D1, c-MET, BCL-2, N-Myc, and CDK4 have been reported to be targets of *miR-34a* [50]. These studies indicate the important role of *miR-34a* in CSCs.

7.5.4 miR-30

Similar to *let-7* miRNA, *miR-30* has been shown to possess a regulatory role in CSCs. Breast CSCs had significant low expression of *miR-30* family members and targets ubiquitin-conjugation enzyme 9 and integrin β3 (Fig. 7.2). Overexpression of *miR-30* in CSCs resulted in inhibition of self-renewal, induction of apoptosis and reduced ability to form tumors and metastasis in immunocompromised mice [50].

7.6 Epigenetic Manipulation of CSC Marker as Therapeutic Treatment for Cancer

Many of the CSCs markers discussed here are overexpressed in various cancers and targeting them results in inhibition of cancer progression. Inhibition of BMI1 using siRNA resulted in inhibition of cancer cell and tumor growth. In ovarian cancer cells, BMI1 siRNA treatment resulted in decreased cell viability and telomerase activity and therefore can be used for treating ovarian cancer. This can also be extrapolated

to other cancers. Inhibition of MSI-1 has been demonstrated to be beneficial for colorectal, medulloblastoma, glioma and breast cancers. Similarly siRNA mediated knockdown of DCLK1 has also been shown to suppress colorectal and pancreatic cancers. Additionally, these CSC markers regulate tumor suppressor miRNAs and inhibition of single gene can result in endogenous overexpression of these miRNAs and ultimately result in inhibition of several oncogenic signaling pathways. Inhibition of DCLK1 resulted in downregulation of Myc and RAS pathway via induction of *let-7a* miRNA, downregulation of EMT via induction of *miR-200a,* downregulation of pluripotency pathway via induction of *miR-143/145* cluster and finally downregulation of Notch pathway via induction of *miR-144*. All of these stem cell related signaling pathways could be controlled or inhibited by targeting a single CSC marker. Development of shRNA and small molecule inhibitors that inhibit these CSC markers are the future of anti-stem cell-based cancer therapy.

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Chapter 8
Recent Updates on Epigenetic Biomarkers for Prostate Cancer

Karen Chiam, Tanya Kate Day, and Tina Bianco-Miotto

Abstract Epigenetics refers to DNA methylation, histone modifications and microRNAs and these epigenetic modifications are extensively investigated as potential biomarkers for cancer. Characterizing genome wide epigenetic changes involved in prostate cancer development and progression will not only identify potential novel therapeutic targets, since some epigenetic modifications are reversible, but also highlight which epigenetic changes can be used as prostate cancer biomarkers. Epigenetic changes are relatively stable and easy to measure in peripheral samples like blood and urine, further highlighting their importance as powerful tools for assessing patient diagnosis and prognosis. In this review, we outline how epigenetic biomarkers have been used for diagnosis, prognosis and for monitoring therapeutic response in prostate cancer. We also review how epigenetic biomarkers may be more sensitive and specific than current prostate cancer serum markers and the possibility that combining different epigenetic modifications may further enhance the diagnostic and prognostic ability of these epigenetic biomarkers. As epigenome wide studies continue to be performed in larger patient cohorts, we will soon identify

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Karen Chiam and Tanya Kate Day contributed equally to this work.

K. Chiam
Cancer Research Program, Garvan Institute of Medical Research
and The Kinghorn Cancer Center, Sydney, NSW 2010, Australia

T.K. Day
Dame Roma Mitchell Cancer Research Laboratories and Adelaide Prostate
Cancer Research Centre, Discipline of Medicine, The University of Adelaide
and Hanson Institute, Adelaide, South Australia 5000, Australia

T. Bianco-Miotto (✉)
The Robinson Institute, Research Centre for Reproductive Health & Early
Origins of Health and Disease, School of Paediatrics and Reproductive Health,
The University of Adelaide, Adelaide, South Australia 5005, Australia
e-mail: tina.bianco@adelaide.edu.au

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the epigenetic modifications involved in prostate tumorigenesis with the resultant identification of new therapeutic targets and robust prostate cancer biomarkers.

Keywords DNA methylation • Histone modifications • MicroRNA • Diagnostic biomarkers • Prostate cancer

8.1 Introduction

Prostate cancer is one of the most commonly diagnosed cancers in men of developed Western countries. Globally, it is the second most commonly diagnosed and sixth leading cause of cancer death in men [1]. Risk factors for developing prostate cancer include family history, race, obesity, diet and other environmental factors. However, age is the best known risk factor for prostate cancer, with 80 % of men developing the disease by 80 years of age [2]. Hence, globally, prostate cancer is a major health and economic burden in the aging population.

When prostate cancer is diagnosed at an early organ-confined stage it is potentially curable by radical prostatectomy, which is the surgical removal of the prostate gland. Radiotherapy is another treatment option that is administered either alone or in combination with radical prostatectomy. However, after initial treatment with curative intent, it has been estimated that approximately 30 % of patients will subsequently relapse with metastatic disease. It is this metastatic prostate cancer, present either at the time of diagnosis or developing after failure of primary therapy, which is the primary cause of mortality from prostate cancer. In the 1940s, it was discovered that prostate cancer is dependent on the male sex hormones androgens for growth and survival. Based on this discovery, therapies targeting androgen production and its mediator, the androgen receptor (AR), were developed. These therapies have been the mainstay for treating patients diagnosed with metastatic disease or progressive disease. Anti-androgens that block the functional action of AR are called hormonal or androgen ablation therapy [3]. Prostate tumors treated with hormonal therapy initially regress in most men, but tumors then become unresponsive to these therapies and progress to the castrate-resistant state after a median time of 18–24 months [4]. In its castrate-resistant state [3], all treatment options for prostate cancer are palliative in nature and have limited benefits in improving patient survival.

8.2 PSA: The Controversial Prostate Cancer Biomarker

The limitations of current prostate cancer treatments highlight a major clinical problem, which is to select the optimal treatment strategy for individual patients at the time of diagnosis. The heterogeneous nature of prostate cancer means that they can be either indolent or aggressive. Patients presenting with the same clinical disease

stage may ultimately have very different outcomes. Since the majority of prostate cancer occurs in elderly men, patients with indolent disease are more likely to die with prostate cancer rather than from the disease. This group of men could avoid treatment altogether, and escape complications and side effects commonly associated with treatment. In contrast, men with aggressive prostate cancer are more likely to benefit and should receive aggressive therapeutic intervention. The ability to discriminate between aggressive and indolent disease at the time of diagnosis will have an extremely positive impact on the quality of life and actual treatment benefits to prostate cancer patients.

Unfortunately, current investigational procedures and prognostic nomograms, which are based on clinical features of the disease, do not accurately identify at diagnosis, patients with disease that is likely to become aggressive and life threatening. Identification of patterns of changes in gene expression, or variations in gene structure or sequence early in prostate tumorigenesis provides an opportunity to define at an early disease stage those cancers that are likely to become life-threatening. The current standard biomarker for detecting and predicting prostate cancer progression is the measurement of serum prostate specific antigen (PSA) level. However, there is constant debate in regards to the efficacy of PSA in the clinical setting for the following reasons [5, 6]:

1. There is no specific cut-off serum PSA level that defines if a patient has prostate cancer. Generally, a high serum PSA level indicates the presence of prostate cancer cells, although it has been shown that a proportion of men with high serum PSA levels do not have prostate cancer [7]. Conversely, approximately 22 % of men with prostate cancer have low serum PSA levels [8]. The false positive and false negative results associated with the serum PSA test means that some men without prostate cancer will unnecessarily undergo an invasive needle biopsy procedure, while in some men their prostate cancer will remain undetected.
2. The serum PSA biomarker is not prostate cancer specific. Increased serum PSA level may be indicative of other prostatic diseases, such as benign prostatic hyperplasia (BPH) and prostatitis. BPH is common in elderly men, with a 75–90 % incidence in men by the age of 80 years, [9, 10] and is therefore a confounding factor in interpreting serum PSA results for some men.
3. Serum PSA levels do not distinguish between indolent and aggressive disease at the time of diagnosis. This is coupled with the fact that the implementation of screening protocols which promotes regular testing of serum PSA levels has resulted in the detection of a high proportion of low stage and low grade prostate cancers. Together, this makes clinical decisions about whether or how to treat the prostate cancer difficult. This is a particular issue for older men with a life expectancy of less than 10–15 years, or men with other medical conditions, who may die from other causes before the prostate cancer becomes a problem for them clinically. In these cases, men and their treating clinicians have to decide between treatment or watchful waiting, which is not an easy decision in the absence of accurate clinical information regarding the likelihood of prostate cancer progression in these men.

4. Monitoring changes in PSA levels can assist clinicians to gauge treatment efficacy. However, this requires ongoing monitoring over a period of time, resulting in a time lag before clinicians can identify if the treatment is working. This means that men may have to receive aggressive treatment such as chemotherapy, with associated unpleasant side-effects, for a prolonged time period before its efficacy can be determined.

Two large clinical trials have recently investigated the effect of serum PSA screening on prostate cancer patients survival in the US (n = 76,693 men) and Europe (n = 182,000 men), with contradicting results reported [5, 6]. There was no significant difference in prostate cancer mortality between patients who underwent annual PSA screening test compared to the control group in the US study, whereas the European study reported a 20 % decrease in prostate cancer mortality associated with PSA screening. A meta-analysis of six randomized controlled trials, including the US and European trials mentioned above, does not support the usefulness of PSA screening on reducing prostate cancer mortality [11].

An ongoing major focus of the prostate cancer research community is to identify better biomarkers or improve current PSA measurements for prostate cancer, yet few biomarkers investigated so far improve upon the diagnostic and prognostic value of serum PSA [12–14]. Two potential prostate cancer biomarkers which warrant further investigation are urinary PCA3 (FDA-approved in February 2012) and urinary TMPRSS2-ERG, both of which aid in the detection of prostate cancer when combined with serum PSA. PCA3 may be helpful in cases where men present with abnormal digital rectal examination (DRE) and/or high serum PSA levels coupled with a negative biopsy. In these cases, a low or negative PCA3 score can be used to determine if a repeat biopsy is necessary or may be avoided [15]. TMPRSS2-ERG gene fusion is the most common gene fusion in prostate cancer, occurring in around 50 % of all cancers [16]. This gene fusion is a potential diagnostic biomarker for prostate cancer detection. A combined measurement of PCA3 and TMPRSS2-ERG may serve as a biomarker of prostate cancer, and is currently under further investigation and validation [17, 18]. Despite these advances, the interpretation of both PCA3 and TMPRSS2-ERG diagnostic biomarkers are still dependent on serum PSA levels. In addition, they are not sufficiently characterized, sensitive or specific to enable their use to predict disease progression or treatment response in the clinical setting.

While the search for prostate cancer biomarkers continues, given the relatively slow progress in this field to date, a new approach is required. There is now rapidly accumulating evidence showing the important contribution of epigenetic modifications to all stages of prostate tumorigenesis [19–22], which may be utilized as novel candidate biomarkers for prostate cancer. In this chapter, we will discuss published studies that have identified and investigated candidate prostate cancer epigenetic biomarkers, as well as the challenges faced in this endeavor and the latest advancements in this research field.

8.3 Epigenetic Biomarkers for Prostate Cancer

Epigenetic alterations are common in prostate cancer and are associated with all stages of tumorigenesis, from initiation to progression of the disease [19–22]. While the exact mechanisms of how these epigenetic changes arise in prostate cancer have not been clearly delineated, they occur at a much higher frequency than mutations, and occur commonly in premalignant stages of the disease [23]. These features make epigenetic modifications attractive biomarkers for diagnosis, prognosis and treatment response (Fig. 8.1).

8.3.1 DNA Methylation Based Biomarkers

DNA methylation is a highly stable epigenetic modification involving the addition of a methyl group to the 5′ carbon of a cytosine residue. This occurs predominantly within cytosine-guanine dinucleotide residues. Since DNA is so stable, analysis is technically relatively simple. Added to this, DNA is found in bodily fluids such as blood, urine and saliva. Medical tests on body fluids are non-invasive and therefore ideal in a clinical setting. All of these factors make DNA methylation biomarkers for prostate cancer attractive for further investigation and discussion.

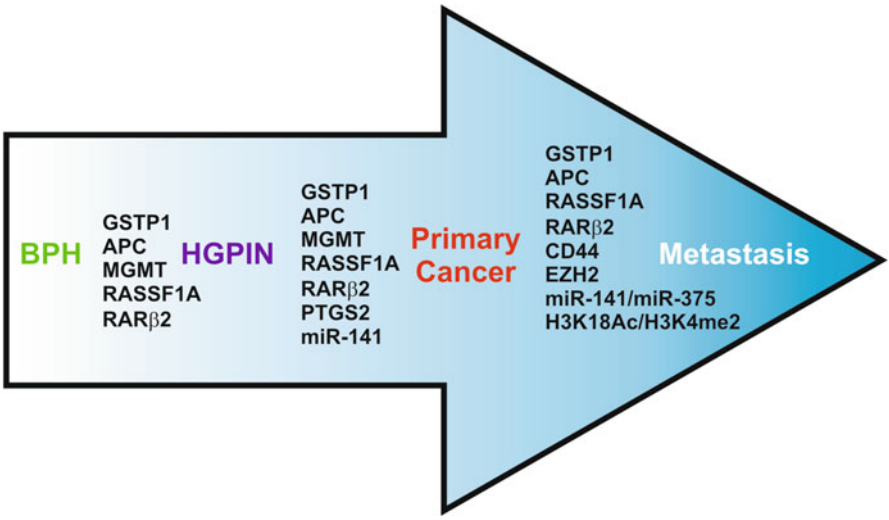

Fig. 8.1 Epigenetic modifications as diagnostic, prognostic and treatment response biomarkers in prostate cancer. Epigenetic alterations that have previously been tested as biomarkers in prostate cancer and cited in the text. BPH refers to benign prostatic hyperplasia and HGPIN refers to high grade prostatic intraepithelial neoplasia

In cancer, global hypomethylation occurs in conjunction with gene-specific promoter hypermethylation [24]. Global hypomethylation is a loss in total genomic DNA methylation, which is linked to activation of proto-oncogenes and chromosomal instability [25, 26]. In prostate cancer, global hypomethylation is associated with metastatic disease [27–30]. An immunohistochemical study performed on human prostate tumor tissues demonstrated a significant decrease in the global 5-methylcytosine levels in patients with recurrent prostate cancer compared to patients without recurrence [28]. Repetitive DNA sequences dispersed in the genome such as retrotransposon elements (i.e. *LINE-1* and *Alu* repeats), which are usually methylated in normal tissues, have been shown to be hypomethylated in prostate cancer [29, 30]. A quantitative methylation-specific PCR (QMSP) study found hypomethylation of *LINE-1* and *Alu* repeats in human prostate adenocarcinoma tissues compared to BPH, and the levels of DNA methylation at these repeat elements correlated with PSA levels and tumor stage [31]. These studies underline the frequency of global methylation changes in prostate cancer. However, none of the above studies investigated if global DNA methylation levels can be used to detect or predict prostate cancer progression. It is only recently that a study has attempted to explore the potential of 5-methylcytosine level to predict survival of patients with prostate cancer [32]. Although a significant decrease in 5-methylcytosine level was observed in the prostate tumors compared to the adjacent normal tissues, there was no association between global DNA methylation levels and patient survival [32].

Up until now, most DNA methylation studies in prostate and other cancers have focused on gene-specific hypermethylation. Gene-specific hypermethylation is an increase in DNA methylation of promoter regions of individual genes. This has been associated with inactivation of genes involved in many cellular functions such as DNA repair, cell-cycle regulation, apoptosis and tumor-suppression [33, 34]. To date, at least 66 genes with promoter hypermethylation have been identified in prostate cancer and is the subject of multiple reviews [20, 21, 35–37]. However, the most frequently occurring and well-studied epigenetic biomarker for prostate cancer is DNA hypermethylation of the glutathione-S-transferase P1 (*GSTP1*) gene promoter.

8.3.1.1 GSTP1 as an Epigenetic Biomarker

GSTP1 encodes an enzyme which is essential for cellular detoxification and protection of DNA from oxidants and electrophilic metabolites [8]. *GSTP1* DNA methylation is an attractive potential epigenetic biomarker for prostate cancer for the following reasons:

1. *GSTP1* DNA promoter methylation is highly specific for prostate cancer (>90 %) compared to serum PSA (~20 %) [38].
2. DNA methylation levels of the *GSTP1* promoter can differentiate prostate cancer from prostatic diseases including BPH and high grade prostatic intraepithelial neoplasia (HGPIN) [39, 40] (Fig. 8.1).

3. *GSTP1* promoter DNA methylation is associated with prostate cancer progression, and disease recurrence after primary therapy [41–43] (Fig. 8.1).
4. *GSTP1* promoter methylation is easily measured in body fluids such as serum, plasma and urine.

There are several published reviews [8, 20, 38, 44–47] highlighting the significance of *GSTP1* hypermethylation as an epigenetic biomarker in prostate cancer. These reviews discussed the techniques and samples (e.g. serum, urine) currently used for analysis and should be referred to for a more detailed insight in this area. Here, we will discuss the features required to further develop *GSTP1* DNA methylation as a robust prostate cancer epigenetic biomarker with utility in the clinic.

Firstly, although *GSTP1* is highly specific for prostate cancer, and more specific than serum PSA, *GSTP1* hypermethlyation does occur in other cancer types. Thus, a key research effort is to enhance the specificity of *GSTP1* as a diagnostic prostate cancer biomarker. One way to achieve this is to measure *GSTP1* DNA methylation in conjunction with a panel of genes with aberrant methylation in prostate cancer [41–43, 48]. The DNA methylation status of a 4-gene panel (*GSTP1*, *RASSF1A*, *RARβ2* and *APC*) has been shown to discriminate prostate cancer patients (n = 95) from age-matched controls (n = 38) with 86 % sensitivity and 89 % specificity [49] (Fig. 8.1). Of particular note, the DNA methylation status was assessed from urine sediments, making this a non-invasive test appropriate for clinical use. Another study demonstrated increased specificity (83–100 %) and sensitivity (94–98 %) when *GSTP1* methylation was combined with *APC* methylation to discriminate between BPH, HGPIN and prostate adenocarcinoma [41] (Fig. 8.1). Using a similar approach, a multi-center study investigated the use of a 3-gene panel (*GSTP1*, *RARβ2* and *APC*) as a diagnostic marker for prostate cancer [50]. The DNA methylation levels of these three genes were assessed by QMSP in the urine samples collected from 337 subjects (178 men with prostate cancer) post DRE and before needle biopsy. The 3-gene panel exhibited an improved accuracy (AUC of 0.57–0.71) compared to serum PSA (AUC of 0.52–0.56), in the detection of prostate cancer [50]. To confirm these findings, the authors performed a similar study in a larger cohort of 704 subjects (320 men with prostate cancer) and demonstrated again that the 3-gene panel (AUC of 0.73) outperformed all other risk factors (i.e. age, serum PSA levels, DRE and family history) (AUC of 0.52–0.66) [51]. These studies demonstrated that as part of a multi-gene biomarker panel, *GSTP1* methylation has great promise as a diagnostic biomarker for prostate cancer diagnosis.

GSTP1 methylation also has potential to act as a prostate cancer prognostic biomarker. The detection of *GSTP1* hypermethylation in patient serum is associated with a 4.4-fold increased risk of biochemical recurrence, measured by PSA relapse [52]. The DNA methylation levels of a 4-gene panel consisting of *GSTP1*, *RASSF1A*, *APC* and *RARβ2* were measured in blood samples from men with prostate cancer, and showed significant association with the risk of biochemical recurrence, although the individual contribution of each gene to this association was not analyzed [53]. In contrast, two other studies have found no correlation between *GSTP1* hypermethylation and biochemical recurrence of prostate cancer [54, 55]; and one study found

that *GSTP1* hypermethylation in human prostate tissue was associated with a decreased risk of biochemical recurrence [56]. The discrepancies among the different studies may be due to differences in characteristics of patient cohorts, methods for DNA methylation analysis and tissue type sampled.

In order to develop an epigenetic biomarker, whether it is an individual gene or a panel of genes, the sample type, timing of collection, and analysis method all require optimization to achieve the greatest sensitivity and specificity. *GSTP1* methylation has been assessed in tissue samples (biopsy or surgically-excised tumor fragments) and also in bodily fluids, including blood, serum, plasma and urine. Assessment using bodily fluids is clearly less invasive and a more desirable option for a biomarker. Wu et al. [38] performed a meta-analysis of over 20 studies, comparing the sensitivity and specificity of *GSTP1* DNA methylation in bodily fluids as a prostate cancer biomarker. *GSTP1* specificity was not influenced by analysis method or sample type; however the sensitivity of *GSTP1* as a biomarker was lower in whole blood and in samples that were collected after treatment, compared to other fluid-based sample types (i.e. plasma and serum). This suggests that for optimal biomarker assessment, samples should be collected prior to treatment, where possible. Obviously, however, if samples are being collected to monitor or measure treatment response, samples need to be collected before and after treatment. Future studies should carefully consider the timing and type of sample collected.

To date, most studies have focused on developing DNA methylation-based markers, including *GSTP1*, as diagnostic and prognostic biomarkers for prostate cancer, and not as a biomarker of treatment response. In principle, in pre-clinical studies and in clinical drug trials, analysis of gene promoter hypermethylation can be employed to assess the efficacy of epigenetic therapeutic agents such as the demethylation agent 5-aza-2′-deoxycytidine (5-aza). Recently, *GSTP1* promoter DNA methylation and re-expression was assessed in human prostate cancer cells after treatment with the demethylating agent 5-aza [57]. *GSTP1* demethylation alone was associated with suppression of cellular proliferation; whereas *GSTP1* demethylation coupled with protein re-expression occurred concomitantly with suppression of proliferation and induction of cell death. Based on this, *GSTP1* presents an attractive target for further testing as a marker of epigenetic therapy response in future clinical trials. In addition to epigenetic therapy response, *GSTP1* has not been investigated as a marker of response to current treatments for prostate cancer, including hormonal therapy and/or chemotherapy. An epigenetic biomarker of treatment response that improves upon the current practice of monitoring serum PSA levels over time, would be of great benefit to patients and their clinicians, by giving information about treatment efficacy earlier in the treatment cycle. An interesting study by Horvath et al. [58] examined methylated *GSTP1* in the plasma of human prostate cancer patients with castrate-resistant disease to investigate if *GSTP1* was predictive of chemotherapy response and survival in these patients. Methylated *GSTP1* levels were measured before and after the first chemotherapy cycle using quantitative methylation-specific head-loop PCR. Patients with decreased methylated *GSTP1* levels after the first chemotherapy cycle were more likely to present a >50 % decrease in PSA levels prior to the fourth chemotherapy cycle (n = 40). Patients with detectable methylated

GSTP1 had a poorer overall survival (23 % survival rate) compared to patients with undetectable methylated *GSTP1* (71 % survival rate) (n = 75), supporting the use of DNA methylation of *GSTP1* as a potential chemotherapy efficacy biomarker for prostate cancer.

8.3.2 Histone Modifications as Biomarkers in Prostate Cancer

Specific histone modifications such as H4K16Ac and H4K20Me3 have been shown to be prognostic in several cancers [59–68]. However, there have only been four studies investigating histone modifications as prognostic markers in prostate cancer [61, 64, 66, 67]. Furthermore, in contrast to the DNA methylation-based biomarkers that have been tested as both diagnostic and prognostic tools for prostate cancer, no study has investigated whether specific histone modifications may be used as diagnostic biomarkers for prostate cancer. The potential of histone modifications as indicators of treatment response in prostate cancer has not been explored and is generally under-studied in all other cancers too, with only two studies (pancreatic and nasopharyngeal cancers) reported in the literature so far [69, 70].

The notion of histone modifications as prognostic biomarkers in cancers was first established in a prostate cancer cohort [61]. Global levels of histone modifications (H3K9Ac, H3K18Ac, H4K12Ac, H3K4Me2 and H4R3Me2) were examined by immunohistochemistry in human primary prostate tumor tissues [61]. With the exception of H3K9Ac, there was a correlation between global levels of all histone modifications and prostate tumor stage [61]. Importantly, the authors demonstrated that combining H3K18Ac and H3K4Me2 predicted tumor recurrence in low grade prostate cancer [61]. A subsequent follow-up study with a larger prostate cancer cohort was able to demonstrate that levels of H3K18Ac and H3K4Me2 were independent predictors of prostate cancer progression regardless of tumor grade [67]. Another histone modification identified to be critical in cancers is H3K27Me3. Loss of H3K27Me3 is common in many cancers and is associated with a poor prognosis [60, 71]. The epigenetic enzyme *EZH2,* which is responsible for H3K27 methylation, is frequently altered during prostate cancer progression and has been shown to be predictive of prostate cancer disease progression [72–78].

The inconsistency and limited studies on histone modifications as cancer biomarkers may be attributed to the lack of technology and methods suitable for the analysis of histone modifications. The most common method used to analyze global expression of histone modifications is immunohistochemistry, which has a relatively low level of sensitivity compared to methods used for DNA methylation analysis. Many experimental factors can also contribute to variations in immunohistochemistry. For example, different antigen-retrieval methods and antibody affinities may affect the immunostaining pattern for a particular histone modification. Most importantly, techniques that can allow accurate measurement of specific histone modifications in body fluids have not been explored, making them less attractive as diagnostic and prognostic biomarkers. The assessment of specific histone modifications in

body fluids is possible following extraction of DNA from serum, plasma or circulating DNA via methods such as ELISA [79, 80]. However, only a single study has utilized ELISA to demonstrate that H3K27Me3 was significantly decreased in metastatic prostate cancer (n = 28) compared to localized disease (n = 33) with an AUC of 0.68 [81]. A recent interesting study investigating the effects of occupational exposure to Nickel on global levels of specific histone modifications (H3K4Me3, H3K9Ac, H3K9Me2) in individuals, has also utilized a similar ELISA approach and found that histone modifications in human peripheral blood mononuclear cells are stable over a period of time [82]. The outcomes of these studies further support the notion of a non-invasive and stable histone biomarker for prostate cancer detection, prognosis and indicators of treatment response may soon be possible.

8.3.3 miRNAs as Biomarkers in Prostate Cancer

An upcoming area of biomarker research in prostate cancer is microRNAs (miRNAs). Studies have shown that miRNAs are of diagnostic and prognostic value for prostate cancer and may even be superior over DNA methylation and histone modifications as biomarkers. Examples of the desirable traits of miRNAs as biomarkers are: they are present and assessable in body fluids (i.e. blood and serum), they are highly stable and have been shown to be tissue- and tumor-specific [83, 84]. Unraveling miRNAs critical in prostate tumorigenesis will subsequently lead to the discovery of novel miRNA-targeted genes and biological pathways implicated in the disease.

Several miRNAs have been identified to be frequently altered in prostate cancer and discussed in reviews [85–87] and in Chap. 3. A collective of studies have shown that distinct miRNA expression profiles can differentiate between non-malignant and prostate tumors, providing evidence that they can be used as diagnostic and prognostic tools [88–95]. For instance, a study by Schaefer et al. [88] undertook a miRNA microarray analysis followed by RT-PCR validation and identified a miRNA expression profile (n = 15 miRNAs) distinct between normal and prostate tumor tissues (n = 76) with an accuracy of 82 %. Of the 15 miRNAs, several were significantly associated with Gleason score (miR-31, miR-96 and miR-205) and tumor stage (miR-125b, miR-205, miR-222) in a second independent prostate cancer cohort (n = 79). High expression of a single miRNA, miR-96, was shown to be associated with increased risk of prostate cancer biochemical recurrence. In another recent microarray study, a miRNA expression profile consisting of 22 miRNAs was able to distinguish between normal and tumor prostate tissues at high prediction rates (91 and 100 % respectively) [89]. In addition, the authors modeled two miRNA expression profiles and investigated them as diagnostic and prognostic biomarkers in the patient cohort used by Schaefer et al. [96]. The modeled diagnostic panel of miRNAs (n = 54) displayed an improved AUC of 0.949 in comparison to that of Schaefer et al. [96]. Most importantly, a separate biomarker panel of prognostic miRNAs (n = 25) displayed an AUC of 0.991 and outperformed Gleason score, pathological stage and serum PSA level in predicting prostate cancer progression [89].

Brase and colleagues [90] generated a profile of the expression of circulating miRNAs (n = 667) in the serum of 21 prostate cancer patients by Taqman miRNA microarray analysis. Further validation of the top five most significantly overexpressed miRNAs (miR-375, miR-9*, miR-141, miR-200b and miR-516-3p) in patients with metastatic compared to localized disease was performed in a separate prostate cancer cohort (n = 45). In this cohort, miR-375, miR-141 and miR-200b were associated with pathological stage and Gleason score. This observation was confirmed in a final validation cohort (n = 71), demonstrating that high expression of miR-375 and miR-141 were significantly associated with pathological stage and Gleason score. The importance of miR-141 and miR-375 as prostate cancer biomarkers was again highlighted by Selth et al. [97], who identified serum miR-141, miR-375, miR-298 and miR-346 levels to be significantly altered in a mouse model of prostate cancer (TRAMP) and in patients (n = 25) with biochemical relapse. High tumor expressions of miR-141 and miR-375 were both significantly associated with increased risk of biochemical recurrence, and miR-375 (HR = 5.70) remained an independent predictor of disease recurrence in multivariate analysis.

8.4 Improvements in Technology and Recent Development of Epigenetic Biomarkers in Prostate Cancer

Identifying methylated DNA requires pre-treatment of DNA, followed by downstream analysis. Historically, the downstream analysis techniques have lent themselves to small scale studies, such as studies of the methylation status of a single gene, or a limited number of candidate genes. Recent technological advances in this area have led to the development of a number of whole-genome methylation techniques, many of which are now broadly accessible and affordable. Coupled with developments in information technology, data from whole-genome epigenetic studies can be integrated with other data sources, opening new doors for the study of epigenetics. In this section, we will discuss some new techniques and concepts in epigenetic study, some of the latest genome-wide studies in prostate cancer, and new epigenetic marks that we consider are likely to make an important contribution to the development of epigenetic biomarkers in clinical prostate cancer.

8.4.1 Genome Wide DNA Methylation: Distinct Profiles and Association with Prostate Cancer Progression

To date, there have been only nine published studies in prostate cancer [98–106] which have utilized at least two independent prostate cancer cell lines and performed an unbiased genome wide analysis of DNA methylation.

One advantage of genome wide methylation analysis is that it is an unbiased technique which can be used to identify methylation of genes or marker DNA regions with potential to act as epigenetic biomarkers of clinical prostate cancer.

Kim and colleagues [103] integrated genome wide DNA methylation results with gene expression, and identified three genes (*PPP1R14C*, *EFEMP1*, *ISL1*) with concordant methylation and expression changes in prostate cancer cells *in vitro*, compared with non-malignant cultured prostate cells. These potential epigenetic biomarkers were validated in clinical samples. *EFEMP1* promoter DNA methylation was the optimal marker to differentiate prostate cancer from BPH, (sensitivity = 95.3 %, specificity = 86.6 %), and this occurred in concert with a reduction in *EFEMP1* gene expression in cancer [103].

Friedlander and colleagues assessed genome wide chromosome copy number, gene expression and DNA methylation changes in metastatic castrate-resistant prostate cancer (CRPC), compared with primary cancer and benign prostate. In this study, 16 genes had concurrent methylation and copy loss in ≥66 % of samples [100], but further validation of these genes as biomarkers of progression was not part of this study. The comprehensive design of this study enabled the authors to demonstrate that DNA methylation changes (10.5 %) occur more commonly than copy number alteration (2.1 %) in CRPC [100]. This observation reinforces the importance of epigenetic biomarkers of prostate cancer, and how they may improve upon PSA serum measurement currently used in clinical practice.

Other genome wide DNA methylation studies have adopted a slightly different approach, and have identified panels of differentially methylated CpGs associated with prostate cancer progression or recurrence. In a clinical cohort, Kobayashi et al. [104] identified a panel of 69 CpGs which were associated with time to biochemical recurrence. These CpGs were located in the promoters of both novel and known cancer-related genes. In the same study, Gleason grade could not be distinguished by DNA methylation profiling. Similarly, Mahapatra et al. [106] analyzed the DNA methylation status of gene promoters and identified panels of genes which were predictive of different types of prostate cancer. A panel of 75 genes could successfully differentiate recurrence from no recurrence, 68 genes could differentiate between systemic recurrence and local recurrence, and 16 genes could differentiate clinical recurrence from biochemical recurrence. A subset of the genes for which promoter DNA methylation was predictive of different types or stages of prostate cancer were validated in an independent clinical cohort. In all cases, this supported the genome wide DNA methylation results, providing further evidence that not only are genome wide techniques highly informative in terms of how many CpGs can be assessed, but they are also accurate and can differentiate between different clinical outcomes or disease stages.

Despite the increasing volume and complexity of data generated, comparisons between studies remain a critical step in selecting biomarkers worthy of further validation and investigation. We sought to determine the degree of similarity between the nine genome wide studies reported to date (Table 8.1). Similarities in genes and gene families identified by genome wide DNA methylation analysis in prostate cancer were assessed. This analysis was somewhat limited by differences in methods, statistical tests used, how the data was presented and made available, clinical versus cell line cohorts, and if the DNA methylation data was combined with gene expression and/or copy number data. Given the multiple sources of technical and biological variation, it was surprising to identify substantial overlap between different

Table 8.1 Genes identified as commonly methylated in prostate cancer by genome wide methodologies

Gene	Cell lines: PCa vs NM	Tumor Vs NM	Progression - Gleason grade	Recurrence
HOXC11	[101]	[106]	[105]	
HOXD3				
HOXD4				
HOXD9				
IRX1				
LBX1				
LHX9				[106]
MNX1				
NKX2		[98]		
SIX6				[106]
VAX1				
AOX1		[104, 106]		
APC	[101, 102]			
BCL2				
C20orf103	[101]		[105]	
CACNA1G	[101, 102]			
CD44				
CDKN2A		[105]		
CYBA		[104, 106]		
ELF4				
FLT4				
GAS6				
GP5	[101]		[105]	
GRASP		[104, 106]		
GRM1	[101]		[105]	
GSTP1	[101, 102]	[104, 106]		
HIF3A				
LAMB3	[99, 101]			
MOBKL2B		[104, 106]		
NEUROG1	[101]			[106]
PYCARD		[104]		
RARB		[104, 106]		
RASSF1	[101, 102]			
RHCG		[104, 106]		
RND2				
RUNX3	[101, 102]	[105]		[106]
SHH	[101]		[105]	
SPATA6		[104, 106]		
SSTR1	[101]		[105]	
TCF7L1				
TFAP2A				
TNFRSF10D				[106]
TPM4		[104, 106]		
WT1	[101, 102]		[105]	
ZNF154		[104, 106]		

studies (Table 8.1). Of note, eight out of nine studies identified genes or gene families overlapping with another study. Only a single study, which had very stringent gene selection criteria and only identified three genes [103], did not have any overlap with any other study. Forty-five genes were identified as differentially methylated in two or more published studies (Table 8.1). As we have discussed earlier in this

chapter, *GSTP1* frequently exhibits prostate cancer-specific gene promoter DNA methylation [107]. This was reflected in the genome wide studies which were included in the comparisons conducted (Table 8.1). The homeobox and T-box gene families (*HOXC11*, *HOXD3*, *HOXD4*, *HOXD9*, *IRX1*, *LBX1*, *LHX9*, *MNX1*, *NKX2*, *SIX6*, *VAX1*) were frequently identified as differentially methylated in cancer compared to non-malignant [98, 101, 106], and during prostate cancer progression [105].

Where studies had more similarities, the number of common genes was higher. For example, focusing on studies which assessed DNA methylation profiles in tumor tissue compared to non-malignant prostate; 16/25 genes identified by Mahapatra et al. [106] were also identified by Kobayashi and colleagues [104]. Similarly, there were many common genes in studies using cell line material (Table 8.1). The degree of similarity also relates to the disease state, with less overlap identified between studies of prostate cancer progression/recurrence than for cell line or cancer versus non-malignant. Taken together, we propose that a general principle is that the degree of variation in DNA methylation between samples is larger than any differences introduced by genome-wide DNA methylation analysis techniques. Therefore, these techniques can provide a reliable and robust measure of genome wide DNA methylation changes. The outcome of our analysis also suggests that, besides the already known gene, *GSTP1*, the homeobox genes may be strong candidates for further development as epigenetic biomarkers for prostate cancer.

8.4.2 The "new" and "under-studied" Epigenetic Marks

The field of epigenetics is constantly expanding and the discovery of 'new-players' creates opportunities for the development of novel biomarkers in cancers. One example is the recent identification of the DNA modification 5-hydroxymethylcytosine, which is a conversion from 5-methylcytosine [108]. Similar to what has been shown with the global loss of 5-methylcytosine in cancers, an immunohistochemical study demonstrated that 5-hydroxymethylcytosine was decreased in prostate cancer [109]. The assessment of both 5-methylcytosine and 5-hydroxymethylcytosine simultaneously may be a better indication of the global levels of DNA methylation. Two other new DNA modifications converted from 5-methylcytosine have also been recently identified, 5-carboxylcytosine and 5-formylcytosine [110], and these warrant further investigation into their roles in prostate cancer and as potential biomarkers.

The advancement in technology is an important factor in the discovery of new epigenetic players in the field. For instance, one recent interesting discovery resulting from the improved technology is the identification of CpG "shores", which are non-CpG islands located outside promoter regions [111, 112]. The methylation status of CpG "shores" demonstrated tissue-specificity and was altered in colon tumors compared to normal colon tissue [111, 112]. Whether this phenomenon of a distinct methylation profile of CpG "shores" may occur in other cancers such as prostate cancer remains to be investigated.

As mentioned earlier (Sect. 8.3.2), histone modifications are under-studied due to the limiting analysis tools to investigate the expression of specific histone modifications in prostate cancer. Genome wide analysis of histone modifications is now possible with methods such as ChiP-sequencing, which allows genomic profiling of multiple specific histone modifications and identifies their interacting proteins that may play an important role in tumorigenesis. One other new technique is the bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq), which allows high throughput DNA methylation to be studied in conjunction with a specific histone modification (i.e. H3K27me3) [113]. The BisChIP-seq technique enables investigators for the first time to analyze the interaction of DNA methylation and specific histone modifications on the same DNA region, which may provide a better interpretation of the subsequent gene expression data readout. This novel technique may be utilized to investigate significant DNA and/or gene regions of concurrent DNA methylation coupled with specific histone modification as potential epigenetic biomarkers in prostate cancer.

Another area which requires further investigation is the potential of histone variants as epigenetic biomarkers in prostate cancer. Histone variants such as γH2A.X and H2A.Z are known to be markers of DNA damage and genomic stability. For instance, γH2A.X is overexpressed in many cancer cell lines including prostate cancer [100], which suggests it is a potential epigenetic biomarker of treatment response to radiotherapy or other DNA damage-targeting drugs. A potential γH2A.X biomarker for such treatment response is desirable due to the ability to measure nuclei γH2A.X in peripheral lymphocytes [114]. The global level of H2A.Z assessed by immunohistochemistry has been demonstrated to be an independent predictor of survival in a breast cancer patient cohort (n = 500) [115]. While no study has investigated whether H2A.Z predicts prostate cancer progression, a study has shown overexpression of H2A.Z levels in a prostate cancer xenograft mouse model [116].

8.4.3 Implication of Epigenetic Biomarkers in Therapy

The availability of a good epigenetic biomarker will undoubtedly aid the development of epigenetic therapy for prostate cancer in various ways. Firstly, epigenetic biomarkers can be used in clinical trials as indicators of epigenetic drug efficacy. For instance, *GSTP1* promoter DNA methylation and re-expression may be a suitable biomarker in clinical trials testing DNA methylation inhibitors in prostate cancer [57]. Although there are FDA approved DNA methylation inhibitors (i.e. 5-aza-cytidine, 5-aza-2′-deoxycytidine) currently used for the treatment of hematological malignancies, clinical trials with these DNA methylation inhibitors have not been as successful in solid tumors. The failure of previous clinical trials has been attributed to inappropriate dose regimens, leading to toxicity-related adverse events. Using a frequent low-dose 5-aza-2′-deoxycytidine regimen, it has been shown that the DNA methylation and protein expression status of *GSTP1* was an indicator of DNA methylation inhibitor (5-aza-2′-deoxycytidine and Zebularine) treatment

efficacy in prostate cancer cells [57]. Hence, future clinical trials involving currently available or new DNA methylation inhibitors in prostate cancer should utilize epigenetic biomarkers such as *GSTP1* (alone or in combination with a panel of genes) to track drug efficacy in patients in a timely manner. However, *GSTP1* DNA methylation has recently been shown to be a marker of response to chemotherapy [58].

Secondly, the identification of epigenetic biomarkers that may have functionally important roles in prostate tumorigenesis can also be potential therapeutic targets. For example, the histone methyltransferase enzyme EZH2, and its substrate H3K27 methylation, are aberrantly expressed in prostate cancer and predict prognosis in several studies (Sect. 8.3.2). Hence, relatively new epigenetic drugs targeting histone methyltransferases and histone demethyltransferases may be potential treatments for prostate cancer. In particular, the histone methyltransferase inhibitor DZNep that inhibits EZH2 activity has been shown to reduce prostate cancer cell growth *in vitro* and *in vivo* [117]. This deserves further investigation and validation of its potential therapeutic use in prostate cancer. We also propose that in future studies, the global levels of H3K27 methylation might be a potential biomarker to determine treatment efficacy for histone methylatransferases like DZNep.

8.5 Future Directions

There is compelling evidence that epigenetic biomarkers for the diagnosis and prognosis of prostate cancer are very promising (Fig. 8.1), but currently, there are few clinical trials investigating these biomarkers for such purposes. From a search in the clinicaltrials.gov database, only three clinical trials were found; two trials investigating a panel of hypermethylated genes in urine and serum as an early detection marker (NCT00340717 and NCT01441687) and a single trial aiming to investigate the association of a miRNA expression profile as a prognostic biomarker (NCT01220427). Several reasons may contribute to the impediment of translating prostate cancer epigenetic biomarkers into clinical trials. Firstly, there is a lack of understanding of the biological significance of these candidate epigenetic biomarkers in prostate tumorigenesis. This is coupled with a lack of consistency in experimental designs to test these biomarkers, and until recently, the limitation of technology available for analysis. Additionally, there are other important factors that should be taken into consideration but have often been overlooked in previous studies investigating the use of epigenetic biomarkers in prostate cancer. For example, since epigenetic alterations arise normally during aging, consideration needs to be made to whether the epigenetic biomarker of interest may also undergo an age-related epigenetic change, especially since prostate cancer is an aging-associated disease.

Nevertheless, with the advancement and availability of state of the art technology for global epigenome analyses, as well as the decrease in the cost of these technologies, the critical epigenetic alterations involved in prostate tumorigenesis will be identified. This will then provide a valuable resource for identifying epigenetic biomarkers that can be used as powerful tools for diagnosis, prognosis and therapy response in prostate cancer.

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Chapter 9
Epigenetics and Racial Disparities in Prostate Cancer

Benjamin A. Rybicki

Abstract Epigenetic changes are drivers of prostate carcinogenesis and may be one biological explanation for racial disparities in prostate cancer incidence and mortality. Surprisingly, despite the growing body of knowledge concerning the role of epigenetics in prostate carcinogenesis, few studies have analyzed methylation differences in prostate cancer by race. To date, the evidence suggests that racial differences in gene methylation patterns in prostate do exist; in benign prostate tissue, African Americans appear to have higher methylation levels of several key genes, such as *APC*, *RARB* and *NKX2.5*, that are known to be involved in prostate carcinogenesis. Whether higher methylation levels in benign prostate translate into a higher prostate cancer risk is unclear, but if one assumes higher methylation levels presage prostate malignancy, then the pre-cancer "field" in prostate defined by methylation may be more primed in African Americans. Only one study to date has examined gene methylation in benign prostate as it relates to cancer risk—however, this study found greater risk in African Americans. In summary, the limited evidence to date suggests that epigenetic changes plays some role in the observed racial disparities in prostate cancer, but more studies are needed to define a broader spectrum of epigenetic profiles in prostate cancer by race—particularly if methylation markers are to have utility as biomarkers and tools for clinical decision making in prostate cancer.

Keywords Racial disparities • Gene promoter hypermethylation • Prostate cancer • Biomarkers • Field cancerization

B.A. Rybicki, Ph.D. (✉)
Department of Public Health Sciences, Henry Ford Hospital, One Ford Place, Suite 3E, Detroit, MI 48202, USA

Population Studies and Health Disparities Program, Karmanos Cancer Institute, Detroit, MI 48202, USA
e-mail: brybick1@hfhs.org

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9.1 Introduction—Is Prostate Cancer Biologically Different in African Americans?

Rates of early prostate cancer detection have increased and survival outcomes have improved in the US over the last 20 years [1], but racial disparities still persist in both incidence and clinical course of the disease. African Americans experience more aggressive disease presentation [2, 3], are more likely to die of prostate cancer [1], and may have higher incidence of prostate cancer after a negative biopsy than Whites [4]. While screening behaviors and access to medical care vary by race [5, 6], a study of prostate cancer mortality among Medicare-eligible men found that social factors explained only 25 % of these disparities [7].

A number of biological markers suggest that androgen biosynthesis and function may vary by race. Mean serum testosterone is higher in African American men under 40 than in White men [8, 9], although this difference diminishes as men age [10]. Androgen receptor expression may also be higher in African American compared with White men [11]. The distribution of prostate specific antigen (PSA) levels also varies across race [3, 12]; among men who do receive prostate cancer screening, established diagnostic protocols may have less efficacy in African Americans, as there is evidence that appropriate interpretation of PSA levels [13] and pathology findings [14] varies by race. Such differences are observed both for men with [3] and without [12] prostate cancer, suggesting that racial differences in underlying prostate biology exist not only for cancer but for other prostate-related conditions.

A recent study found that prostate cancer volume after radical prostatectomy was greater in African American than in White men, and that metastatic disease was four times more common [2], suggesting that differences in the biological presentation of prostate cancer—such as growth rate or transformation to more aggressive phenotype—likely influence racial disparities in diagnosis and mortality rates. Several studies have demonstrated differences in gene expression in prostate tumors from African American and White men, particularly in pathways associated with immune response [15, 16]. Other studies have found racial differences in copy number variation in tumors. Rose et al. [17] found significant enrichment of copy number alterations in genes related to immune response. Our own research group recently analyzed DNA copy number changes in prostate tumors and found no significant differences between prostate tumors of White and African Americans in overall mean allelic balance, combined loss, or copy-neutral events [18]. Further analyses of these data using a copy number biomarker validated in Whites [19] found that 80 % of African American patients positive for this biomarker went on to develop biochemical recurrence; however, the majority of African American patients in our study population (82 %)—including many patients that experienced recurrent disease—were biomarker-negative, resulting in a low negative predictive value of 61 %. Such findings emphasize that, while a biomarker optimized in a White population may have predictive utility in some African Americans, additional discovery is often needed to identify biomarkers with wider applicability across all ethnicities.

If the biology of prostate cancer differs by race, then it is reasonable to consider whether patterns of DNA methylation in prostate also vary between African American and White men. Unfortunately, little is known about the prostate cancer methylome in African Americans, but the existing studies that have examined racial differences suggest that gene methylation patterns in prostate may indeed differ by race [20–25] emphasizing the importance of studying race-specific methylation patterns; in fact, our own work suggests that the relationship between methylation of the *APC* and *RARB* genes and risk of prostate cancer varies by race [23].

Characterization of tumor suppressor genes inactivated by hypermethylation—and the timeline when such changes occur—may improve early prostate cancer detection and disease management, which could have a significant impact upon racial disparities. In the chapter that follows, we discuss what is known about racial differences in gene methylation on a population level, and in the female counterpart of prostate cancer, breast cancer. Next, we discuss how methylation plays a role in all stages of prostate carcinogenesis. We then conduct a comprehensive review of the existing literature regarding racial differences in DNA methylation in prostate cancer, highlighting our recent work regarding racial differences in prostate cancer risk associated with methylation in the benign prostate. We discuss differences in methylation patterns in a key gene, *RARB*, that has been studied extensively in prostate cancer risk methylation studies, and how CpG regions interrogated across studies influence results. We finish with some closing thoughts on deficiencies in our current knowledge of prostate methylation as it relates to racial disparities and key areas that should be areas of focus for future studies.

9.2 Racial Differences in Methylation—Leukocytes and Breast Cancer Tissue

Much research has emphasized how DNA methylation patterns vary among cell types and across time; however, methylation patterns may also be inherited, and remarkably only two studies have investigated how inherited epigenetic patterns vary by race, and whether methylation differences present at birth are associated with later differences in the incidence and mortality of cancers. One of these investigated CpG sites associated with cancer-related genes [26] whereas the other investigated methylation more broadly [27]. Adkins et al. [26] studied whole blood acquired at birth, finding that methylation patterns in 13.7 % of autosomal CpG islands differed between African American and Whites. In regions where methylation was significantly associated with race, they found a 1.8-fold ($p = 0.00139$) enrichment of 31 genes involved in the prostate carcinogenesis. In contrast, Zhang et al. studied methylation levels in leukocytes from 161 cancer-free adult subjects; they observed a 2.2 % lower level of leukocyte *LINE-1* methylation in non-Hispanic African Americans versus Whites [27]. The authors did not offer any explanations for the lower levels of methylation in African Americans and noted that since African Americans carry the lowest frequency of the methylation disrupting

MTHFR C677T polymorphism, the opposite result would be expected. The main methodological difference between the Adkins et al. and Zhang et al. studies in terms of methylation and cancer is that the former measured methylation at CpG sites biased towards cancer-related genes whereas the latter was a more general measure of global methylation.

Like prostate cancer, breast cancer presentation varies by race, and shares pathogenic pathways (such as sex hormone sensitivity) with prostate cancer [28, 29]. Although overall breast cancer incidence is higher in White women, incidence prior to age 50 is higher and the disease is more deadly in African American women. A study of methylation in invasive ductal carcinomas found similar levels of methylation across groups—except in (estrogen/progesterone-receptor negative) tumors from African American women younger than 50. These tumors displayed significantly higher methylation levels in four genes (*HIN-1*, *Twist*, *Cyclin D2*, and *RASSF1A*) involved in apoptosis and tumor suppression [30]. Another study, using cluster analysis of methylation levels in 773 cancer-related genes, found unique methylation profiles across races [31]; the majority of tumors from African American women were in a cluster associated with greater tumor size and younger age at presentation.

A study of candidate-gene CpG island methylation in breast cancer found differences between African American and White patients in the *CDH13* gene; these differences were even more pronounced among younger patients and those with estrogen receptor-negative disease [32]. The *CDH13* tumor suppressor gene produces a protein that mediates cell-cell interaction and cancer cell invasion and metastasis [33]; the authors hypothesized that hypermethylation of this gene may contribute to racially-distinct molecular alterations contributing to early onset of breast cancer. The study also found three loci (*RASSF1A*, *RARB,* and *CDH13*) that were methylated more often in breast tumors from African American women; methylation of these genes was associated with poor prognoses, suggesting that differences in methylation patterns may contribute to more aggressive disease among African Americans. Methylation of certain genes has also been associated with more aggressive disease and poor outcomes in prostate cancer, but no studies exist that have directly tested gene methylation as a potential confounding factor for racial differences in prostate cancer outcomes.

9.3 Preneoplastic Methylation Changes in Prostate Carcinogenesis

Prostate cancer is characterized by its marked multi-focal nature—67–96 % of prostatectomy specimens display more than one tumor focus [34, 35]. Hanson et al. [36] first showed that gene promoter methylation occurs in the non-neoplastic cells of the prostate tumor microenvironment. Troyer et al. [37] found that 84 % of men with a methylated *RARB* gene had a subsequent diagnosis of prostate cancer upon repeat biopsy. Steiner et al. found that the same *RARB* gene studied by Troyer et al.

was often found methylated in normal prostate cells located as far as 10–20 mm from the primary tumor [38]. We recently found that men with *RARB* methylation in benign prostate had an increased risk for subsequent prostate cancer that persisted almost 10 years after initial benign biopsy, but this increased risk while nominal in Whites was consistently greater than two in African Americans.

The multifocal nature of prostate cancer and methylation changes in histologically appearing benign prostate tissue that either becomes malignant after a short time or is adjacent to cancer suggests the presence of a 'field effect,' whereby factors underlying carcinogenesis result in molecular changes in areas beyond the tumor foci. Epigenetic change is a potential measure of a generalized field effect in the prostate [39]. As described above, such changes are measurable in histologically normal cells and often precede overt carcinogenesis. Another example is gene expression patterns of histologically benign tissue adjacent to tumor that more closely resembles a malignant gene expression phenotype than that of normal prostate cells [40]. In a sample of prostate cancer cases that was 60 % African American [41], expression of the let-7 family of miRNAs in histologically normal prostate tissues from Gleason grade 7 or higher tumor was decreased compared to histologically normal tissue from Gleason grade 6 tumors. Further analysis of expression data comparing the two adjacent normal specimens from grade 6 and 7 tumors with normal tissue controls found only the normal adjacent tissue from grade 6 tumors was similar to the normal tissue controls suggesting normal prostate tissue adjacent to grade 7 tumors has already undergone a molecular malignant transformation. Investigation into whether this field cancerization effect is more pronounced in African American cases may provide insights about why African Americans have more aggressive prostate cancer.

9.4 Methylation Changes in Prostate as Markers for Progression

Methylation of specific genes at different stages of prostate carcinogenesis provides insight into potential carcinogenic pathways. DNA methylation status may also serve as a biomarker for prostate cancer detection and disease progression. As described above, methylation changes in prostate tissue often occur before histological changes are evident; methylation changes are also more common and consistent than many somatic genetic changes. Thus, identification of methylation markers that occur throughout the course of prostate carcinogenesis may also provide insight into pathogenic pathways that are targets for intervention.

For instance, prostatic intraepithelial neoplasia (PIN) [42] is the precursor lesion of prostate cancer; in these lesions, methylation in the gene *GSTP1* is common [43, 44]. Other genes—including *RARB*, *APC*, *MGMT*, and *RASSF1A*—display increasing methylation as carcinogenesis progresses [44–47]. In benign prostate tissue, *RARB* is more often methylated than *APC* [23, 44], but *APC* methylation may serve as a pre-malignant marker with high negative predictive value for subsequent

prostate cancer [48]. *APC* methylation is also strongly associated with high-grade tumors [23] and shown to be an independent predictor of poor prognosis [49, 50]. In the most comprehensive study to date of whole genome methylation changes associated with different prostate cancer phenotypes, Mahapatra et al. [47] measured methylation status in 14,495 genes. They found methylation of different genes associated with presence of disease and disease recurrence—genes such as *GSTP1* and *RARB* were associated with prostate cancer incidence, while genes such as *BCL11B* and *RASGRF2* were associated with systemic recurrence.

Aberrant methylation changes may arise from any number of insults to the prostate, incidental or cumulative [51]. Such changes appear consistently in prostate cancer; for example, methylation of the *GSTP1* gene is the most reliable biomarker of the disease [52] and researchers have identified over 30 genes hypermethylated in prostate cancer [53]. These include tumor-suppressor genes, genes involved in hormone responses, tumor-cell invasion, cell cycle control, and DNA damage repair. Changes occurring in the early stages of tumor development are homogeneous and persist through the progression of the disease—these may serve as biomarkers for early detection. However, methylation patterns in recurrent tumors are heterogeneous—suggesting that prostate tumor cells acquire distinct epigenetic changes as they progress. A key question with regard to prostate cancer risk and racial disparities is whether early and/or late epigenetic changes associated with disease outcomes follow a race-specific pattern.

9.5 Genes Displaying Racial Variation in Methylation in Prostate

While publications regarding methylation and prostate cancer now number in the hundreds [53], those including race as a factor are far less common (Table 9.1). The first reported study that examined racial differences in prostate cancer methylation studied hypermethylation of the *GSTP1*, *CD44*, and *E-Cadherin* genes in tumor tissues of African American and White prostate cancer patients [25]. Although *GSTP1* methylation was found in most tumor tissues (~84 %), there were no differences across groups. In contrast, *CD44* was methylated 70 % more frequently ($p = 0.05$) in African American than White cases; methylation frequency also correlated with tumor grade. A later study by this same group examined racial differences in methylation for eight genes (*GSTP1, RASSF1A, RARB, CD44, EDNRB, E-cadherin, Annexin-2*, and *Caveolin-1*) [20]. Methylation of *CD44* actually showed a stronger association with race (OR (odds ratio) = 2.0 versus 1.7), but none of the eight genes showed a statistically significant difference in terms of methylation percentages by race. It should be noted, however, that the sample size decreased from 111 to 90, reducing statistical power compared to the previous study.

Methylation in the gene *GSTP1* is highly specific [52] for prostate cancer. A study of *GSTP1* by Enokida et al. investigated whether promoter hypermethylation

Table 9.1 Summary of studies that have examined racial differences in prostate cancer-related gene methylation

Study	Sample			Tissue type	Genes analyzed	Genes with significant results	Notes
Woodson et al. [25]		**AA**	**W**	FFPE from RP, TURP, or needle biopsy	*GSTP1; CD44; E-cadherin*	*CD44*	1.7-fold higher frequency of *CD44* methylation in AA (43 vs. 25 % in AA vs. W; P = 0.05)
	Cases	47	64				
	Controls	NA	NA				
Woodson et al. [20]		**AA**	**W**	FFPE from RP, TURP, or needle biopsy + cell lines	*GSTP1; RASSF1A; RARB2; CD44; EDNRB; E-cadherin; Caveolin-1; ANXA-2*	*CD44*	Methylated in 41.5 % (AA) vs 26.5 % (W), P = 0.10
	Cases	41	49				
	Controls	NA	NA				
Enokida et al. [22]		**AA**	**W**	FFPE from RP (cases); TURP (BPH, controls)	*GSTP1*	*GSTP1*	When complete *GSTP1* methylation present, HR = 13.4 (AA) vs 3.8 (W)
	Cases	44	77				
	Controls	38	38				
Das et al. [21]		**AA**	**W**	RP (cases); TURP (BPH, controls) + cell lines	*PYCARD*	*PYCARD*	*PYCARD* methylated more in AA cases, but only a risk factor in W cases (OR = 7.6)
	Cases	21	45				
	Controls	12	22				
Kwabi-Addo et al. [24]		**AA**	**W**	RP (paired tumor/normal) (cases); needle biopsy (controls)	*GSTP1; AR; RARB; SPARC; TIMP3; NKX2.5;*	all except *GSTP1*	Methylation of *RARB* and *NKX2.5* increased more rapidly with age in African Americans
	Cases	39	113				
	Controls	18	13				
Tang et al. [23]		**AA**	**W**	Benign biopsy & TURPs (case-control pairs), RP (cases)	*RARB, APC, MGMT, RASSF1, CCND2*	*RARB, APC*	*RARB* methylation a risk factor only for AA (OR = 2.09); *APC* methylation a risk factor for high grade disease
	Cases	211	300				
	Controls	211	300				

Abbreviations: *AA* African Americans, *W* Whites, *FFPE* fixed formalin paraffin embedded, *RP* radical prostatectomy, *TURP* transurethral resection of the prostate, *BPH* benign prostatic hypertrophy

of this gene correlated with clinico-pathological findings in a mixed-race (Asian, African American, and White) sample of 291 prostate cancer cases. The researchers also compared methylation percentages from these cases with 172 benign prostate hypertrophy specimens [22], using assays specific to each of the two sites necessary for functional *GSTP1* promoter activation [54]. In Whites, the ratio of positive methylation results from at least one assay for prostate cancer versus BPH was 2:1; in African Americans, it was almost 6:1. This difference increased further when limited to positive results from both assays (i.e., more complete methylation across the *GSTP1* promoter region); the ratio was 4:1 in Whites, but 13:1 in African Americans. These results clearly demonstrate how gene promoter methylation in prostate cancer may vary by race.

A similar study of 66 prostate cancer and 34 BPH tissue samples investigated methylation patterns in the *TMS1/ASC* (aka *PYCARD*) gene [21], known to play a role in apoptosis [55]; methylation of this gene is associated with breast cancer [56]. Interestingly, the authors found that *TMS1/ASC* methylation was more prevalent in prostate cancer cases than controls in White patients (OR = 7.6; p 0.002) while no difference between the cases and controls was seen in African American patients. A subsequent analysis of five additional genes known to be methylated in prostate cancer—*GSTP1, CD44, ECAD, RASSF1A* and *EBR*—did not reveal any statistically significant differences in methylation by race. As in previous studies, however, the modest number of specimens limited the statistical power; although the risk associated with *RASSF1A* methylation was much higher for African Americans than Whites (OR = 8.6 vs. 3.2) none of the risk estimates reached statistical significance.

Perhaps the most comprehensive study of racial differences in prostate cancer methylation by race was performed by Kwabi-Addo et al. [24]. These authors used pyrosequencing to quantitatively measure the methylation status of *GSTP1, AR, RARB, SPARC, TIMP3*, and *NKX2-5* in prostate tumor and normal tissue specimens from African American and White patients to assess differences in methylation by age and race. Overall, they observed significant methylation differences by race after adjusting for cancer status. Tumor specimens from White patients displayed slightly higher Gleason score and similar pathologic staging when compared with the African American samples. Thus, the higher prevalence of methylation seen in the African American cancer samples was not simply an artifact of differences in disease aggressiveness or stage between the two groups. In addition, regression analysis revealed significantly higher age-adjusted methylation levels for *NKX2-5* and *TIMP3* genes in the normal prostate tissue samples of African American cases. Of the six genes that were analyzed in the normal prostate tissue samples, methylation of *NKX2-5* also showed modest evidence for a race-by-age interaction, suggesting that this gene may also be a more sensitive methylation biomarker in African Americans.

While this study provided evidence that methylation levels of key genes in the prostate vary by race, the authors were unable to demonstrate that these methylation differences translated into prostate cancer risk differences [24]. Although receiver operator characteristics analyses showed suggestive racial differences in

the predictive potential of DNA methylation for the *GSTP1, RARB, SPARC, TIMP3*, and *NKX2-5* genes, the study was underpowered to demonstrate statistically significant differences by race. Despite this shortcoming, the results of Kwabi-Addo et al. raised the possibility of designing "ethnic-sensitive" biomarkers for prostate cancer detection.

Notably, the four studies reviewed above all compared the methylation status of genes in prostate tumor tissue of cases with that of benign prostate tissue of controls; such cross-sectional study designs do not provide insight into temporal associations between methylation events and prostate carcinogenesis. Our own research was designed to address this limitation by nesting a case-control study within a longitudinal cohort of men with benign prostate specimens; we tested. the association of methylation of five tumor suppressor genes, *MGMT, RASSF1, RARB, APC and CCND2* in benign prostate tissue with risk of subsequent prostate cancer [23]. We also measured methylation in a subset of paired benign-tumor specimens to validate that methylation of *RARB* and *APC* in prostate are stable events in the prostate carcinogenesis pathway.

In our sample of 211 African American and 300 White case-control pairs, the methylation-associated prostate cancer risk varied by race; a positive association between *RARB* methylation and prostate cancer risk was found only in African Americans (OR = 2.09; p = 0.002) while a negative association for methylation of the *MGMT* gene was observed only in Whites (OR = 0.50; p = 0.03). When cases were stratified by tumor grade (low vs. high), the highest risk estimates were observed in African Americans; the association of methylation of *APC* with high grade tumors was greater in African Americans than Whites (OR = 3.21 vs. 2.04). In African Americans, *APC* and *RARB* methylation appeared to act in concert to increase risk, particularly after adjusting for PSA level and presence of high-grade PIN (OR = 3.04; p = 0.003). The same was not true for whites, with the joint OR for *RARB* and *APC* methylation only slightly elevated (OR = 1.14; p = 0.7) and significantly different from that observed in African Americans (p = 0.01). Our results showed that in African Americans, methylation of the *RARB* and *APC* genes that occurs before histological evidence of disease are biomarkers of subsequent disease risk.

Across the studies that have investigated racial differences in prostate cancer methylation, methylation results in 15 genes have been reported (Table 9.2). Most have been investigated in only one study, and therefore any significant findings—such as variation in aged-related *NKX2.5* methylation by race [24] or a positive association between *RARB* and *PYCARD* methylation and prostate cancer risk limited to African Americans [21, 23]—require replication. Although most of the observed racial differences show higher levels of methylation in African Americans, some exceptions exist; for example, two studies have both shown higher levels of *RASSF1A* methylation in benign prostate of Whites compared with African Americans [21, 23]. Results are also not consistent across all studies: while Enokida et al. found significantly higher risk associated with *GSTP1* methylation in African Americans [22], this finding was not replicated by two other studies [21, 24]. Likewise, the higher methylation levels of *CD44* in prostate tumors of African Americans reported by Woodson et al. [20, 25] were not replicated by Das et al.

[21]. Of the associations listed in Table 9.2, the most consistent are for *RARB*. This is likely due to both the targeted region of methylation that has been studied as well as the critical role this gene plays in prostate carcinogenesis.

9.6 An Exploration of *RARB* Methylation Differences by Race

Retinoic acid receptor-beta (*RARB*) is a tumor suppressor gene on chromosome 3p24, where a high incidence of loss of heterozygosity is detected in many types of tumors. Retinoic acid suppresses cancer cell growth through binding to retinoic acid receptors (RAR), especially RAR-beta. Selective loss or down-regulation of RAR-beta mRNA and protein has been reported in prostate cancers [57], and the distinct cellular distributions of RAR subtypes in benign, pre-neoplastic, and malignant prostate tissues suggest links between altered RAR signaling and deregulated cell growth and carcinogenic processes [58]. *RARB* methylation is a sensitive and specific marker for prostate cancer [37], and appears to increase in the course of prostate malignancy [59]. As described above, levels of *RARB* methylation in normal prostate tissue are higher in African American than White men [23, 24].

The study by Adkins et al. of CpG methylation in leukocytes collected at birth examined four *RARB* CpG loci; one of the four—cg26124016—exhibited significant methylation differences between African Americans and Whites [26], with an average methylation percentage of 3.5 % in African Americans and 5.7 % in Whites ($p = 10^{-12}$). This locus resides within the *RARB* promoter region, about 300 base-pairs upstream of the transcription initiation site.

This same *RARB* promoter region (Fig. 9.1) has been highly interrogated by three studies that have reported racial differences in *RARB* methylation [20, 23, 24]. There are 21 CpG loci spanning 200 base-pairs within the *RARB* gene promoter region and exon 1; for the 13 of the 21 CpG loci where methylation was measured in one or more of the three studies, the results have been remarkably consistent. In overlapping CpG loci residing in *RARB* exon 1, Tang et al. [23]and Kwabi-Addo et al. [24]both reported 34–37 % methylation in benign prostate tissue and 63–67 % methylation in tumor tissue of African Americans, versus 23–27 % methylation in benign and 59–68 % methylation in tumor tissue of Whites. Both studies found lower methylation levels in benign prostate tissue specimens from Whites than African Americans (Fig. 9.2), although Kwabi-Addo et al. found slightly higher *RARB* methylation in prostate tumors of African Americans (67 % vs. 59 %) while Tang et al. found the opposite (63 % vs. 68 %). Neither study could report statistically significant racial differences in *RARB* methylation in prostate tumors. Woodson et al. [20] reported similar results in their investigation of methylation levels in prostate tumor tissue for four CpG loci upstream of the transcription initiation site (Fig. 9.1).

If racial differences in *RARB* methylation occur in benign prostate tissues, these differences apparently disappear when the prostate becomes malignant. While the clinical profile of the prostate cases among the above studies varied (Kwabi-Addo et al.

Table 9.2 Summary of genes tested for methylation differences by race in prostate

Gene	Gene product	Cancer pathway	Tumor	Racial variations		CpG Island	References
				Normal	Risk		
APC	Adenomatous polyposis	Tumor suppressor		√	√	Promoter/exon 1	[23]
AR	Androgen receptor	Hormone receptor gene; metabolism of androgens and testosterone				Promoter/exon 1	[24]
CCND2	Cyclin D2	Cell cycle regulation				Promoter	[23]
CD44	CD44 antigen	Lymphocyte activation; recirculation and homing; tumor metastasis	√			Promoter/exon 1	[20, 21, 25]
ERRFI1	ERBB receptor feedback inhibitor 1	Cell growth and signaling				Exon 2	[21]
CDH1	E-cadherin protein	Adhesion molecule—loss of function increases cell proliferation, invasion and/or metastasis				Exon 1	[20, 21, 25]
EDNRB	Endothelin receptor type B	Cell growth and signaling				Promoter	[20]
GSTP1	Glutathione S-transferase P1	DNA repair			√	Promoter	[21, 22, 24]
MGMT	O(6)-methylguanine-DNA methyltransferase	DNA repair				Exon 1/intron 1	[23]
NKX2.5	Homeobox protein	Gene transcription	√	√		Exon 1	[24]
PYCARD	PYD and CARD domain containing	Inflammatory and apoptotic signaling pathways		√	√	Promoter	[21]
RARB	Retinoic acid receptor beta	Gene transcription		√	√	Promoter/exon 1	[20, 23, 24]
RASSF1	Ras-association domain family 1A	Tumor suppressor		√		Promoter/exon 1	[21, 23]
SPARC	Secreted protein, acidic and rich cysteine	Cell-matrix interactions; migration and angiogenesis	√			Exon 1	[24]
TIMP3	Tissue inhibitors of metalloproteinase inhibitors 3	Degradation of extracellular matrix	√	√		Exon 1	[24]

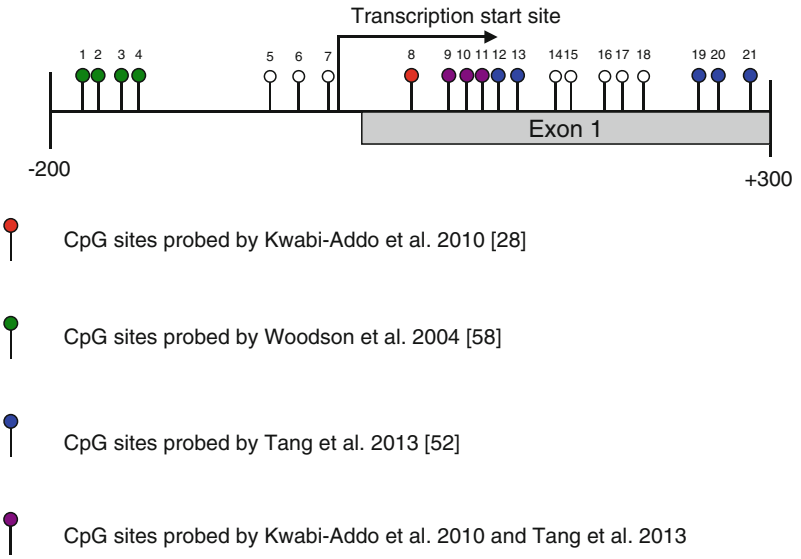

Fig. 9.1 CpG loci spanning a 500 base pair region across the *RARB* promoter and exon 1 where racial variation of methylation status in prostate has been previously studied. The region in question is located on chr3: 25469522-25470022 and contains 21 CpG loci

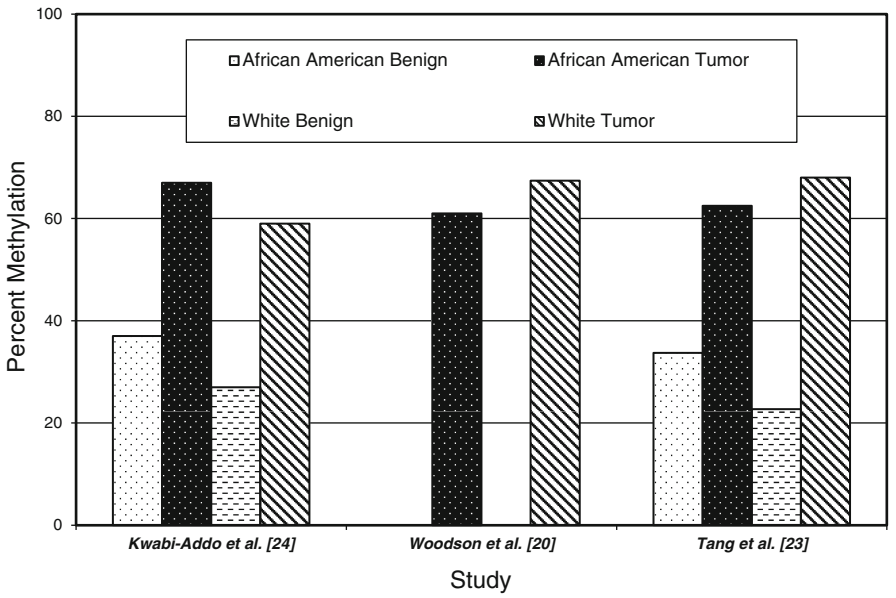

Fig. 9.2 Racial variation in *RARB* methylation levels in benign prostate and prostate cancer across three studies

included more advanced stage cases, but tumors of lower Gleason grade than Woodson et al. or Tang et al.), these clinical differences do not appear to be associated with *RARB* methylation. Nor did the choice of which CpG loci were queried—suggesting that methylation changes occur over a broad span of the *RARB* promoter region and likely occur in the earliest stages of carcinogenesis. If high levels of *RARB* methylation are a precursor for prostate cancer, then the elevated *RARB* methylation observed in histologically benign prostate tissue from African American men [23, 24] may suggest a higher baseline risk for this racial group; this is consistent with epidemiologic observations. As we noted previously from our work [23], *RARB* methylation in benign prostate is associated with prostate cancer in African Americans but not in Whites—this further underscores the importance of considering race in studies of epigenetic change and cancer.

9.7 Conclusions

Can gene methylation and its effect on prostate carcinogenesis explain some of the racial disparities observed in prostate cancer? In prostate tumors, it does not appear that methylation levels vary significantly by race for most genes. However, given that methylation across a field of cells happens gradually and the current thinking that certain epigenetic events might help define the "cancer field" [39], it is conceivable that racial differences in both baseline methylation levels of key cancer-related genes within benign prostate as well as the rate at which these genes become methylated with age might affect overall prostate cancer risk. Our understanding of racial differences in gene methylation in prostate is based on only a few studies, and more work is clearly needed to define race-specific epigenetic profiles of prostate cancer—particularly if methylation markers are to have utility as biomarkers of disease presence and as tools for clinical decision making. Ideally, future studies will be racially diverse and will include whole genome methylation surveys [47] to explore the full range of methylation marks in prostate tissue that may vary by race. While the concept of race is complex and involves both social and biologic constructs [60], as the field of epigenetics becomes richer and its role in molecular medicine grows, we can expect to learn more about the impact of aberrantly-methylated genes upon prostate cancer risk and how this risk is manifest across the full spectrum of men affected by this disease.

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Chapter 10
Epigenetic Signatures of Breast Cancer Genes

Shrikant Anant, Animesh Dhar, and Satish Ramalingam

Abstract Breast cancer is a leading malignancy among women with higher western countries, suggesting significant role for environmental factors in developing breast cancer. Recently, epigenetic modifications such as aberrant methylation and acetylation of genes and histones have been shown to play a critical role in breast cancer development. There are several articles published in the recent years with the major epigenetic signatures of breast cancer genes. Therefore compiling these information could lead to a greater understanding of the development of breast cancer and novel approaches for chemoprevention. Here we have provided different modes of epigenetic regulation including DNA methylation, histone modification, polycomb group of proteins, and non-coding RNAs. In addition, we have provided information on chemotherapeutic drugs that act through regulation of epigenetics and have progressed to clinical trials. Most importantly, we have analyzed the epigenetic regulation in the chemotherapy resistant breast cancer stem cell population. Furthermore, the epigenetic regulatory mechanisms of various breast cancer related genes are discussed in detail. Taken together, in this review we have discussed the current understanding of the modes of epigenetic regulation, and the epigenetic signatures seen in breast cancer.

S. Anant (✉) • S. Ramalingam
Department of Molecular and Integrative Physiology, University of Kansas School of Medicine, Kansas City, KS, USA

University of Kansas Medical Center, University of Kansas School of Medicine, Kansas City, KS, USA
e-mail: sanant@kumc.edu

A. Dhar
Department of Molecular and Integrative Physiology, University of Kansas School of Medicine, Kansas City, KS, USA

Cancer Biology, University of Kansas School of Medicine, Kansas City, KS, USA

University of Kansas Medical Center, University of Kansas School of Medicine, Kansas City, KS, USA

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Keywords Methylation • Acetylation • Histone modifications • MicroRNA • HDAC • Jumanji

10.1 Introduction

Breast cancer is a leading malignancy among women, and has long evaded attempts at prevention. In spite of early detection and improved treatment, every year nearly 250,000 women discover that they have invasive breast cancer [1]. Roughly 58,000 more will be diagnosed with early cases of the disease and about 40,000 will die [2]. The incidence of breast cancers is much higher in western countries than that in other parts of the world, suggesting a significant role for environmental factors in developing breast cancer. Genetic and epigenetic alterations have both been shown to play an important role in breast carcinogenesis. The former is irreversible while the epigenetic changes are reversible. Epigenetic malfunctions are manifested through aberrant methylation and acetylation of genes and histones involved in normal tissue development, leading to activation or silencing gene expression. Thus numerous molecular events could go awry because of epigenetic malfunctions. The advancement in the science of epigenetics has led to a greater understanding of how breast cancer forms, resulting in the discovery of novel approaches for chemoprevention. Specifically, identifying DNA methyltransferases and histone deacetylases that control epigenetic modifications has resulted in utilizing these enzymes as primary targets for epigenetic therapy. In this review, we will discuss the current understanding of the modes of epigenetic regulation, and the epigenetic signatures seen in breast cancer.

10.2 History of Epigenetics

Epigenetics is the study of changes in phenotype or gene expression caused by mechanisms other than those that alter the DNA sequence. In 1942, Conrad Waddington coined the term epigenetics; he derived the name from a Greek word epigenesis, a theory of development [3]. In 1969, Griffith and Mahler were the first to suggest that DNA methylation might have an important biological role on gene expression, and that changes in DNA methylation might explain how genes are turned on and off [4]. A few years later, in 1975, Sager and Kitchin proposed that there are enzymes in eukaryotic organisms that restrict unmodified DNA [5]. Since then it has become apparent that changes in DNA methylation might play important role during carcinogenesis [6]. More recently, this has expanded to other types of modification, including histone modifications [7].

10.3 Types of Epigenetic Regulation in Breast Cancer

The interesting aspect of epigenetic alteration of DNA is that such changes are heritable but does not alter nucleotide sequence. This is in contrast to a genetic change where the nucleotide changes. Furthermore, unlike genetic changes, epigenetic modifications are potentially reversible; this aspect gives the potential for therapy against cancer. The science of epigenetics has explained how nutrients and drugs can change the cancer cell cycle [8, 9]. Epigenetics causes the organism's genes to behave differently, such as the changes seen when cells differentiate or become malignant.

Different modes of epigenetic regulation have been observed in breast cancers including (1) DNA methylation (stable and long term repression), (2) histone modification (dynamic and can be changed upon stimulation), (3) polycomb group of proteins (maintain the silenced state of developmental regulators), and (4) Non-coding RNAs (microRNA, small nucleolar RNA, repeat-associated small interfering RNA) (Fig. 10.1). In this chapter, we will describe each one of these regulatory mechanisms (Table 10.1).

10.3.1 DNA Methylation

DNA methylation, a major mode of epigenetic regulation occurs on cytosine residues of CpG dinucleotides [10]. DNA methylation affects the packing of chromatin and the architecture of the nucleus thereby critically regulating gene expression.

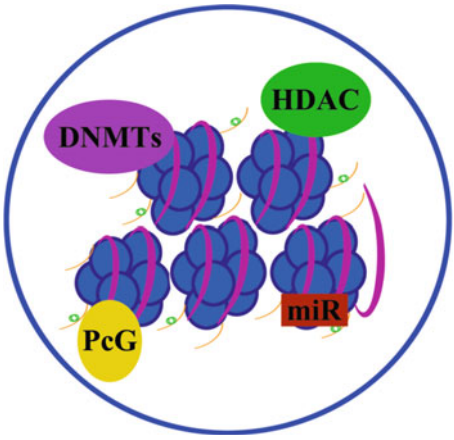

Fig. 10.1 Major modes of epigenetic regulations include DNA methylation, histone modifications, Polycomb Group proteins, and microRNA. DNA Methyltransferases (*DNMTs*) adds methyl group to the cytosine and inhibits transcription, while Histone deacetylases (*HDAC*) along with Polycomb Group proteins and microRNA forms a repressive complex to inhibit gene transcription

Table 10.1 Modes of epigenetic regulation

Modification	Mechanism
DNA methylation	Occurs on cytosine residues of the CpG dinucleotides in DNA
Histone modification	Covalent modification of the N-terminal of certain amino acids on the histone tails
Poly Group Proteins	Forms repressive complex for inhibition of transcription
MicroRNA	Gene silencing, and by triggering transcriptional silencing via chromatin remodeling

CpG islands are regions of at least 500 bp and have more than 55 % GC content [11]. CpG islands have mostly been identified within promoter regions, and methylation within this region makes the DNA inaccessible and no longer recognizable by the transcriptional machinery resulting in gene silencing. A methyl group is added to the cytosine ring by DNA methyltransferases by an enzymatic transfer from the methyl donor S-adenosylmethionine to the carbon-5 position of cytosine [12]. Surprisingly, according to Antequera and Bird, approximately half of all genes in the human (~45,000 genes) contain CpG islands [13]. Various studies have clearly shown that about 70–80 % of CpG sites in the human genome are methylated. DNA-cytosine methyltransferase-1 (DNMT1) was the first methyltransferase to be identified [14]. Subsequent studies have suggested that the DNA methyltransferase 1 (DNMT1) plays a key role in the maintenance and restoration of methylation after DNA replication, while DNMT3A and DNMT3B initiate *de novo* methylation and establish new DNA methylation patterns [15].

In general, normal cells are hypomethylated, while in cancer cells hypermethylation is frequent leading to silencing of tumor suppressor genes. Retinoblastoma gene was characterized to be the first tumor suppressor gene and also they are the first to be identified as hypermethylated. The importance of promoter hypermethylation has been characterized using demethylating agents 5-aza-2′-deoxycytidine to reactivate the genes in cancer cell lines. DNA methylation has been shown to play important role in normal development e.g. development of fertilized egg into an embryo. Apart from this, nearly 45 % of the human genome has repetitive sequences and loss of methylation of these sequences is thought to account for most of the global hypomethylation observed in all human cancers. Hypermethylation of the CpG promoter regions, in the tumor suppressor genes, results in gene silencing and therefore resulting in the oncogenic transformation. These changes have been shown to account for various cancers including breast cancer development. Methylation inactivates the transcription; demethylation may result in transcriptional interference and dysregulation of normal gene expression, leading to destabilization and chromosomal translocations.

There are several genes that are silenced by promoter methylation during breast cancer development. Here, we have attempted to comprehensively provided the list of genes that are hypermethylated at the promoter region during breast cancer development.

10.3.2 Cell Signaling Genes That Are Hypermethylated During Breast Cancer

10.3.2.1 Secreted Frizzled-Related Proteins (SFRPs)

Wnt signaling plays a significant role in development of various types of cancer and inhibiting the pathway hinders the progression of tumorigenesis [16]. Secreted frizzled-related proteins (SFRPs), a family of proteins that include SFRP1 to SFRP5, are extracellular antagonists for Wnt signaling [17]. These proteins are shown to sequester Wnt molecules at the cell surface membrane and thereby regulate the Wnt signaling pathway during breast cancer development [18]. Several reports have also suggested that increased nuclear and cytoplasmic accumulation of β-catenin is due to disruption of SFRPs and Wnt equilibrium in the breast tumor tissues [19]. Subsequent studies have demonstrated that SFRP1 and SFRP5 genes are targets for the promoter hypermethylation leading to inactivation [20, 21]. Moreover, promoter hypermethylation is associated with unfavorable prognosis in breast cancer.

10.3.2.2 Estrogen Receptor (ER)

Estrogens are hormones that play a key role in the growth and development of breast cancers. Estrogens mediate its action through intracellular estrogen receptors (ER) [22]. There are two estrogen receptors ER-α and ER-β, which are encoded by ESR1 and 2 genes, respectively [23]. Estrogen receptors can also function as transcription factors to regulate the expression of target genes. In breast cancers, ER-α methylation is a predictive marker for response to hormone therapy [24]. Importantly, up to a third of the breast cancers lack ER-α at the time of diagnosis and a significant proportion of cancer patients who are initially ER-α positive become ER-α negative during tumor progression [25]. Initially it is thought that these changes are due to genetic alterations such as deletion or mutation; however, recent studies have demonstrated that there is DNA methylation in the promoter region [26]. The ER-α gene, located at chromosome 6q25.1, has CpG rich regions in both its promoter and the first exon [27]. While these CpG rich regions are unmethylated in normal breast tissues and in ER-positive breast cancer, it is methylated in ~50 % of the unselected primary breast cancers and most of the ER-negative breast cancer cell lines [28]. However, treatment with methyltransferase inhibitors such as 5-aza-cytidine (5-aza-C) and 5-aza-deoxycytidine (5-aza-dC) results in partial demethylation and restoration of ER mRNA expression and protein [29]. This has been further confirmed by various studies where it was observed that DNMT1 expression at both RNA and protein levels in ER-negative breast cancer cell lines are significantly increased compared with their ER-positive counterparts.

10.3.2.3 Retinoic Acid Receptor β2 (RARβ2)

RARβ2 gene is a member of the nuclear receptor superfamily [30]. There are six receptors (RARα, -β and -γ and retinoid X receptors-α, -β and -γ) in this family and all of them are ligand activated transcription factors [31]. RARβ gene, located at chromosome 3p24, has been implicated in playing an important role in limiting the growth of various tumors, including breast cancer [32]. Methylation of CpG islands in the RARβ2 promoter region is one of the factors linked to downregulation of its expression in breast cancer [33]. Furthermore, methylation of the RARβ promoter has been shown in several RARβ2-negative human breast cancer cell lines and in about one-third of unselected primary breast cancer specimens, a result of which is decreased or complete loss of RARβ2 expression [34]. However, treatment with 5-aza-dC can partially restore RARβ2 expression further confirming the promoter methylation mediated suppression of its transcription [35].

10.3.2.4 Aplasia Ras Homolog Member I (ARHI)

The Ras-related novel tumor suppressor gene aplasia Ras homolog member I (ARHI; also known as DIRAS3) encodes a small GTPase with 60 % homology to Ras and Rap. The expression of this tumor suppressor is down regulated in 40 % of DCIS and 70 % of invasive breast cancers [36]. Furthermore, reexpression of ARHI protein suppresses clonogenic growth of breast cancer cells, inhibits their invasiveness, and induces apoptosis. There are three CpG islands in the ARHI gene. The first two CpG islands are located in the promoter region, while the third CpG island is located in the coding region [37]. Furthermore, hypermethylation of the second CpG island within the promoter region results in the complete loss of ARHI expression in breast cancer cells [38].

10.3.3 DNA Damage Response Genes That Are Methylated During Breast Cancer

10.3.3.1 BRCA1

The BRCA1 gene located at chromosome 17q21, is a well-known breast cancer susceptibility gene [39]. Inhibition of BRCA1 expression through antisense oligonucleotides increases the proliferation of normal and malignant mammary cells while overexpression of wild-type BRCA1 suppresses MCF-7 breast cancer cell tumorigenesis in mice [40]. Inherited mutations in the BRCA1 gene account for one-half of inherited breast carcinomas. However, in contrast to other tumor suppressor genes, somatic mutations in this gene have not been reported, despite the high degree of LOH at the locus in breast cancers [41]. Subsequent studies of the BRCA1 promoter region demonstrated increased methylation in 11 % of sporadic breast cancer cases, which was inversely correlated with expression of both ER and

progesterone receptor [42]. Additionally, BRCA1 promoter methylation is associated with medullary and mucinous subtypes [43]. Moreover, while BRCA1 was unmethylated in normal tissues and in various breast cancer cell lines, two xenograft studies from two of these cell lines demonstrated increased promoter methylation and the concomitant loss of transcript [44]. In addition, the study demonstrated that loss of heterozygosity could happen through the aberrant methylation of the second allele. Finally, BRCA1 methylation is observed only in breast and ovarian cancers but not in tumors of colon or liver or leukemia, supporting a tissue-specific event for the process.

10.3.3.2 ANKRD11

The ankryin repeat domain-containing protein ANKRD11, a co-activator for p53 is a putative tumor suppressor gene in breast cancer. Downregulation of ANKRD11 is associated with breast tumorigenesis [45]. The promoter region of ANKRD11 is loaded with CpG-rich regions, and analysis of this region demonstrated that high levels of methylation in 40 % of breast tumors but not in normal breast tissues or normal blood samples. Moreover, treatment of breast cancer cell lines with DNA demethylating agents results in upregulation of ANKRD11 expression suggesting that promoter DNA methylation plays a key role in downregulating the protein expression.

10.3.3.3 Mismatch Repair Genes MLH1 and MSH2

Loss of genomic stability is associated with a variety of diseases, particularly cancer. Of the many proteins that maintain genomic integrity, two important ones are the mismatch repair genes MLH1 and MSH2 [46]. Analysis of these mismatch repair genes in locally advanced breast cancers demonstrated that hypermethylation of the MLH1 gene in 43.5 % of patients with primary breast cancer, of whom 66.9 % had locally advanced breast cancer (stages IIIA to IIIC) [47]. Similarly, there was hypermethylation of the MSH 2 gene in 16 % of primary breast cancer cases. Of these patients, 21.3 % had locally advanced breast cancer [48].

10.3.4 Cell Cycle and Apoptosis Genes That Methylated During Breast Cancer

10.3.4.1 Ras Association Domain Family 1A Gene (RASSF1A)

RASSF1A gene, located within the 120 kb 3p21.3 minimal homozygous deletion region is epigenetically inactivated in cancers, suggesting a tumor suppressor function for the protein [49]. The protein is 55 % homologous to Nore1, a noncatalytic protein identified by its ability to bind selectively to active Ras [50]. Forced ectopic

expression of RASSF1A in cancer cells reduced colony formation, suppressed anchorage-independent growth and inhibited tumor formation in nude mice [51]. More importantly, methylation in the RASSF1A gene is highly correlated with breast cancer risk, atypical cytology and benign breast disease requiring biopsy [52]. With respect to age, RASSF1A gene methylation has been noted to increase linearly between the ages of 32 and 55 [53]. In microdissected breast tissue, Lehmann et al. showed that the RASSF1A promoter was methylated in all epithelial hyperplasia and papilloma samples and in 83 % of ductal carcinoma in situ, suggesting methylation of RASSF1A promoter as a new marker for nonphysiological epithelial proliferation in the breast [54]. The study also found that in most cases of progression to invasive growth, epigenetic inactivation takes place before invasive growth develops, an observation confirmed by Pasquali et al., who observed a progressive gain of methylation for RASSF1A from normal to hyperplasia acquiring statistical significance at CIS and invasive carcinoma [55]. Functionally, one thing that RASSF1A does is it influences G1-S cell cycle checkpoint by regulating the levels of cyclin D1 protein. Reduced RASSF1A expression due to epigenetic silencing leads to accumulation of cyclin D1, which may represent an important mechanism for overriding cell cycle control under conditions of increased cell cycle pressure [56].

10.3.4.2 Cyclin D2 (*CCND2*)

CCND2 is a member of the D-type cyclins, implicated in cell cycle regulation, differentiation, and malignant transformation [57]. It should be noted that cyclin D2 is not expressed in the majority of breast cancer cell lines, whereas abundant expression can be detected human mammary epithelial cells with a finite lifespan [58]. Also, loss of CCND2 expression caused by methylation is an early event in breast cancer tumorigenesis. Hypermethylation of the CpG island in the promoter can be detected by methylation-specific PCR in most breast cancers, and this has been associated with silencing of cyclin D2 gene expression. Promoter hypermethylation was also detected in ductal carcinoma *in situ*, suggesting that loss of cyclin D2 expression is an early event in tumorigenesis [59]. Furthermore, methylation of CCND2 has been correlated with poor prognosis, implying that CCND2 has a tumor suppressor function.

10.3.4.3 Cyclin D/CDK4 Complex Inhibitor (CDKN2/p16)

Frequent LOH and homozygous deletion has suggested the presence of tumor suppressor genes in chromosome 9p21 [60]. One such gene in this locus is inhibitor of the cyclin D/CDK4 complex (CDKN2/p16), which is frequently deleted in human cancer cell lines. The protein binds to CDK4 and CDK6, and blocks Gl to S transition by inhibiting cyclin-D-dependent phosphorylation of the Rb protein and

maintains its binding to the E2F transcriptional factor [61]. Since the initial reports of homozygous deletion, numerous studies have shown varying, but in general much less frequent, abnormalities of p16 in primary tumors of these cancers. For example, although the rate of homozygous deletions ranged from 40 to 60 % of breast cancer cell lines, neither homozygous deletion nor point mutations were observed frequently in primary breast carcinomas [62]. Furthermore, certain neoplasms, such as prostate and colon cancer, have not been found to harbor homozygous deletions in established cell lines. However, *de novo* methylation of the 5′ CpG lsland is a frequent mode of suppressing CDKN2/p16 expression, and also firmly demonstrate that CDKN2/p16 is one of the most frequently altered genes in human neoplasia.

10.3.4.4 Retinoblastoma Protein-Interacting Zinc Finger Gene RIZ1

RIZ was first isolated in a functional screening for Rb-binding proteins, and was also independently isolated as DNA-binding protein MTB-Zf, GATA3 transcription factor-binding protein G3B, and a coactivator of estrogen receptor. RIZ contains the canonical Rb-binding motif LXCXE and the nuclear hormone receptor-binding motif LXXLL [63]. In addition, RIZ contains a novel protein methyltransferase domain, called the PR domain or SET domain, which is present in ~50 human genes [64]. Two products of the gene exist: (a) RIZ1, which contains the PR domain; and (b) RIZ2, which lacks the domain. RIZ1 but not RIZ2 has tumor-suppressive properties [65]. The *RIZ1* gene maps to chromosome 1p36, a region commonly deleted in more than a dozen different types of human cancers. RIZ1 expression, but not RIZ2 expression, is commonly silenced in many types of human tumors, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma through DNA methylation. Further studies are required to determine specific sites of methylation in the gene.

10.3.4.5 TMS1

TMS1 belongs to a growing family of apoptotic signaling molecules that contain a CARD domain [66]. This domain is found within the prodomain of a number of caspases, and oligomerization with upstream CARD-containing regulatory proteins mediates their cleavage and activation. Other CARD-containing proteins with known roles in apoptosis include the Caenorhabditis elegans CED-3 and CED-4, the human homologue of CED-4, apoptotic protease activating factor-1, the cellular and viral inhibitors of apoptosis, the cellular homologue of herpes virus EHV2 E10 protein, BCL10, and several proteins involved in the activation of NF-kB. The protein was found to act during the initiation phase of an apoptotic pathway, coupling death receptors at the cell surface or intrinsic death signals to the activation of the caspase cascade. Earlier studies suggest that the gene encoding TMS1 is silenced by DNA methylation [67].

10.3.4.6 14-3-3-σ

14-3-3-σ is a member of a superfamily of proteins that are responsible for instituting cell cycle checkpoint in response to DNA damage [68]. Several lines of evidence have demonstrated that hypermethylation of the 14-3-3-σ gene occurs in 91 % of primary breast cancers and is strongly associated with loss of 14-3-3-σ gene expression. Hypermethylation of the 14-3-3-σ gene occurs in a CpG-rich region that extends from the transcriptional initiation site to the middle of the coding region. Bisulfite genomic sequencing of the 500-bp region showed that it is consistently and densely methylated in cell lines and primary breast tumors. This dense methylation just downstream of its transcriptional start site is strongly associated with gene silencing. Further proof of methylation silencing was obtained when treatment with 5-aza-dC resulted in demethylation of the CpG island and reactivation of gene expression [69]. Moreover, 14-3-3-σ expression is undetectable in 94 % (45/48) of breast tumors. Subsequent studies have clearly demonstrated that CpG island methylation is the epigenetic event that is largely responsible for silencing of the 14-3-3-σ gene and occurs in a majority of breast cancers.

10.3.5 Other Genes That Are Hypermethylated in Breast Cancers

10.3.5.1 Death-Associated Protein Kinase (DAP-Kinase)

DAP-kinase is a 160-kDa serine/threonine, microfilament-bound kinase recently shown to be involved in γ-interferon-induced apoptosis [70]. Tumor invasiveness and aggressiveness has been associated with hypermethylation of a CpG island in the promoter region of the gene. In a small series of B-cell malignancies, a similar finding of methylation of the DAP-kinase CpG was also described, suggesting that hypermethylation of the DAP-kinase gene and loss of γ-interferon-mediated apoptosis may be important in the development of B-cell malignancies [71]. Cell lines derived from these cancers also demonstrate reduced or DAP-kinase gene expression, and reexpression can be seen following treatment with a DNA demethylating drug such as 5-aza-dC [72].

10.3.5.2 ID4

ID4 is the most recently discovered member of the Inhibitor of DNA binding/Inhibitor of differentiation family of transcription factors [73]. ID proteins contain a helix-loop- helix (HLH) domain enabling interaction with other basic helix-loop-helix (bHLH)-proteins. Following heterodimerization with those transcription factors, ID proteins act as dominant negative inhibitors of gene transcription [74]. In addition, ID proteins can bind to other important non-bHLH transcription factors such as the retinoblastoma protein (RB) or the paired box (PAX)-proteins, thereby regulating

important pathways in cell proliferation and differentiation [75]. Furthermore, ID4 is an important factor for the development of the nervous system. In this regard, the ID4 gene is highly expressed in migrating postmitotic neurons, in Purkinje cells, as well as in the adult cerebellum. Since ID proteins regulate fundamental cellular processes, a link of ID dysregulation with human carcinogenesis has been recently postulated. ID1 and ID2 are overexpressed in several human tumor cancers including pancreatic cancer and colorectal adenocarcinomas, suggesting a putative oncogeneic function for these two proteins [76]. However, ID3 and ID4 expression is reduced in several tumor types such as ovarian adenocarcinomas. In human breast tissue ID4 mRNA was found to be constitutively expressed in normal mammary epithelial cells, but suppressed in estrogen receptor ER-positive breast carcinomas and pre-neoplastic lesions. A human ribozyme library-based inverse genomics approach revealed that ID4 might act as a negative regulator of the common tumor suppressor gene BRCA1. Moreover, ID4 expression levels were decreased in BRCA1/ER-positive breast cancer specimens, suggesting that ID4 participates in molecular events regulating ER and BRCA1 expression. Recently, it has become very evident that promoter methylation plays a decisive role in the expression of these genes in cancer. Aberrant hypermethylation of the ID4 gene promoter was associated with an increased risk for lymph node metastasis [77].

10.3.5.3 Paired-Like Homeodomain Transcription Factor 2 (PITX2)

PITX2 acts as a transcription factor and are shown to regulate procollagen lysyl hydroxylase gene expression. Furthermore, this protein is involved in the development of organs [78]. Hypermethylation of the PITX2 promoter is associated with a high risk of recurrence in node-negative, steroid hormone receptor-positive breast cancer following tamoxifen adjuvant therapy [79]. In addition, DNA hypermethylation of eight candidate genes (BMP4, LMX1A, BARX1, FGF4, NR5A1, LHX4, ZNF1A1, and CCND2) linked to the PITX2 signal transduction pathway was found to be significantly associated with patient outcome [80].

10.3.5.4 Adenomatosis Polyposis Coli (APC)

The APC gene product modulates β-catenin function by binding to the protein and driving it to ubiquitin-proteosomal degradation [81]. Genetic and epigenetic alterations in APC, a tumor suppressor gene originally implicated in colon cancer have been reported in many malignancies including breast cancers. A study of 76 breast cancer patients by Liu and colleagues has demonstrated that APC gene methylation correlated positively with TNM staging and negatively with protein expression [82]. Lee et al. reported that methylation occurs in the APC promoter in 42 % of breast cancer aspiration fluid samples. However, the gene was unmethylated in the aspiration fluids from normal breast tissue in patients with breast cancer and all benign breast disease patients in their cohort [83].

10.3.5.5 GSTP1

Glutathione (GSH) and its corresponding cytosolic GSTs are involved in the detoxification pathway of xenobiotics and chemotherapeutic agents [84]. They catalyze intracellular detoxification reactions by conjugating chemically reactive electrophiles to GSH, inactivating electrophilic carcinogens. The GSTs, encoded by several different genes at different loci, have been classified into α, μ, π and θ families. The π-class GST, encoded by the GSTP1 gene, on chromosome 11, is of particular importance in breast cancer [85, 86]. In cultured breast cancer cell lines an inverse relationship between GSTP1 and ER gene expression has been reported, i.e. GSTP1 was expressed in ER-negative but not in ER-positive lines, although the underlying mechanism is unclear. Treatment of the GSTP1-negative cell line MCF-7 with 5-aza-dC induces *de novo* synthesis of π-class protein. In addition, GSTP1 promoter methylation has been associated with gene inactivation in about 30 % of primary breast carcinomas, and this correlates with PR expression. It is postulated that methylation-associated inactivation of GSTP1 can result in adenine or guanine mutation by estrogen metabolites-DNA adduct formation and lead to genetic instability [87].

10.3.5.6 TIMP3

TIMP3 belongs to a family of molecules that inhibit the proteolytic activity of matrix metalloproteinases [88]. The protein can suppress primary tumor growth *via* its effects on tumor development, angiogenesis, invasion and metastasis. TIMP3 is methylated in ~30 % of human breast cancer cell lines and also in ~30 % of primary breast tumors [89]. Hypermethylation of 5′CpG island in TIMP3 promoter has been observed in normal and benign (ALH) lesions and in DCIS and tumor lesions, implicating TIMP3 as an early event. Furthermore, 5-aza-dC treatment has been shown to induce TIMP3 expression supporting a role for epigenetic mechanism in TIMP3 gene regulation [90].

10.3.5.7 Progesterone Receptor (PR)

Progesterone receptor (PR) is a classical estrogen-regulated gene [91]. Receptor status is important in classification of breast cancers. The PR gene encodes two isoforms, hPRA (79 kDa) and hPRB (109 kDa), which differ in both their N-terminal sequences and biological activities. The PR gene, located at chromosome 11q13, also has a CpG island in its first exon [92]. PR gene methylation has been demonstrated in ~40 % of PR-negative breast tumors and PR-negative breast cancer cell lines [93]. Additional proof of the functional role for CpG island methylation was obtained when treatment of PR-negative MDA-MB-231 cells with 5-aza-dC in the presence of estrogen led to partial demethylation of the PR CpG island and re-expression of PR gene [94].

10.3.5.8 E-Cadherin

The E-cadherin gene, located at chromosome 16q22.1, encodes a cell-surface adhesion protein that is important in maintaining cell–cell adhesion in epithelial tissues [95]. Considerable evidence shows that loss of expression and function of E-cadherin protein contributes to increased proliferation, invasion and metastasis in breast cancer. This also correlates with decreased patient survival. While mutations and deletions clearly play a role in loss of the E-cadherin expression and function, several studies have also demonstrated that epigenetic silencing of the E-cadherin gene by 5′CpG methylation occurs in human breast cancer cell lines as well as about 50 % of unselected primary breast cancers [96]. Recent studies have demonstrated that hyper methylation of the E-cadherin CpG island was evident in about 30 % of ductal carcinomas *in situ* and increased significantly in nearly 60 % of metastatic lesions [97].

10.3.5.9 LAMA3

Interaction between epithelial cells and extracellular matrix is important for the structural integrity and specialized function of breast epithelium. Two way signaling occurs *via* extracellular proteins (laminins) and their transmembrane receptors, the integrins. Hemidesmosomes are structures used by normal epithelia to adhere to basement membrane. The major structural proteins of the hemidesmosomes are the integrins and its ligand laminin 5 (LN5). In lobular and ductal structures of the breast, both myoepithelial and luminal cells has hemidesmosomes. LN5 is specific to epithelium, and it is a heterotrimeric protein member of the laminin family and consists of three polypeptide chains α3, β3, and γ2, which are the products of three different genes LAMA3, LAMB3, and LAMC2. The chains are assembled in a coiled cruciate-like structure, which is deposited in the basement membrane [98]. Silencing of LAMA3 gene by methylation plays an important role in pathogenesis of breast cancers [99].

10.3.5.10 Klotho

Klotho is a single pass transmembrane protein, associated with premature aging and acts as a potent inhibitor of insulin receptor [100]. Its expression is reduced associated during breast cancer development. Methylation of its promoter region and silencing of its expression has been reported to occur at the early stages of breast cancer [101].

10.3.5.11 Slit2

The Slit family comprises large ECM 3 secreted and membrane-associated glycoproteins [102]. Human Slits (SLIT1, SLIT2, and SLIT3) are candidate ligands for

the repulsive guidance receptors, the ROBO gene family. The SLIT2 gene has been mapped to chromosome 4p15.2, and studies have shown that the putative SLIT2 receptor, is methylated in some breast tumors [103]. In addition, Slit2 promoter hypermethylation is detected in tissue and serum samples from breast cancer patients [104] Furthermore, ectopic expression of SLIT2 in several breast cancer cell lines suppressed growth and reduced colony formation abilities.

10.3.5.12 Caveolin-1 (Cav1)

Cav1 is a ubiquitous scaffolding protein that coats plasma membrane invaginations termed caveolae in various cell types [105]. The Cav1 gene is located in the locus D7S522 of human chromosome 7q31.1, a region that is frequently deleted in human cancers, implicating Caveolin-1 as a tumor suppressor [106]. Aberrant promoter methylation of Cav1 gene is associated with reduced of expression, and occurs at the precancerous stage [107].

10.3.5.13 Lost-On-Transformation 1 (LOT1)

LOT1 is a growth suppressor gene localized on chromosome 6 at band q24–25, which is a frequent site for loss of heterozygosity in many solid tumors [108]. The gene encodes a nuclear transcription factor and is strongly regulated by the activation of the epidermal growth factor receptor-signaling pathway [109]. Earlier studies have identified CpG islands in the upstream sequences of exon 1 and in the promoter region, suggesting the potential for DNA methylation [110]. In addition, recent reports have demonstrated that the gene is located within a maternally imprinted chromosomal region, and the gene is methylated. Moreover, breast cancer cell lines have high levels of CpG methylation in its promoter region.

10.3.5.14 Sulfotransferase 1A1 (SULT1A1)

SULT1A subfamily of sulfotransferases is an important phase II xenobiotic metabolizing enzyme that mediates the sulfonation of drugs, carcinogens, and steroids [111]. Specifically, SULT1A1 plays a significant role in the sulfoconjugation of xenobiotics, such as p-nitrophenol, N hydroxy-heterocyclic and -aromatic amines, and endogenous compounds such as di-iodothyronine and estrogens. The CpG methylation rate of the SULT1A1 gene in breast cancer was shown to be denser than in normal and benign tissues [112].

10.3.5.15 Cystatin E/M (CST6)

Cystatin M or E/M (encoded by the CST6 gene) is an endogenous inhibitor of lysosomal cysteine proteases that functions to protect cells against uncontrolled

proteolysis. CST6 has been shown to be involved in the degradation of components of connective tissues and basement membranes *in vitro*, and aberrant expression and activity of these proteases accompany cancer invasion and metastasis *in vivo* [113]. Absence of CST6 expression is believed to result in increased proteolysis of tissue architecture, facilitating the spread of cancer cells. Studies have established a strong link between CST6 promoter hypermethylation and loss of CST6 expression in breast cancer [114].

10.3.5.16 WW Domain Containing Oxidoreductase (WWOX)

WWOX is a tumor suppressor gene spanning a genomic region of f1 Mb located at chromosome 16q23.3-24.1, a region with a high incidence of loss of heterozygosity (LOH) in breast, prostate, and other cancers [115]. The area is highly methylated in breast cancers, resulting in reduced gene expression. Similarly, a significantly greatly reduced levels of WWOX gene expression was observed in MDA-MB-231 cells, which was determined to be partly due to the methylation of the CpG islands [116].

10.3.5.17 Dickkopf-3 (DKK3)

Dickkopf genes (DKK) encode a class of extracellular signaling molecules. Together, they control cell fate during embryonic development and regulate tissue homeostasis in adults. There are four DKK members, DKK1–DKK4. While DKK1, DKK2 and DKK4 antagonize canonical Wnt/β-catenin signaling at the membrane, DKK3 functions in antagonizing nuclear β-catenin levels. DKK3 expression is frequently lost in human cancer tissues because of aberrant 5′-cytosine methylation within its promoter region including in breast cancers [117].

10.3.5.18 CCAAT/Enhancer Binding Protein D (CEBPD)

C/EBPs are a highly conserved family of leucine zipper proteins that regulate cell growth and differentiation in multiple organs [118]. Specifically, C/EBPδ functions to initiate and maintain growth arrest of mammary epithelial cells [119]. Loss of function alterations in C/EBPδ gene expression has been reported in human breast cancer and in rodent carcinogen-induced mammary tumors [120]. This has been associated with C/EBPdelta gene promoter hyper- and site specific-methylation [121].

10.3.5.19 Deleted in U Twenty Twenty (DUTT1)

DUTT1is a member of NCM family of receptors was mapped at human 3p12 [122]. DUTT1 protein consists of an ectodomain of five Ig domains and three-fibronectin type III repeats. It also contains a single transmembrane segment and a long cytoplasmic domain that does not contain any recognizable motifs except proline-rich

repeat and sequences of low compositional complexities as predicted by the SMART program. The gene is frequently deleted in breast cancer. In addition, the gene can be hypermethylated in breast cancer and its expression reactivated by 5-aza-dC treatment [123].

10.4 Histone Modification

Histones are proteins that assemble and have DNA wrapped around them. There are four histones, with the core histones present as octamers (2 each of 4 core histones) as well as two linker histones H1 and H5. For many years it has been known that post-translational modifications of histone tails determine, in part, which regions of the genome are an open and thus transcriptionally active conformation, and which are closed and thus transcriptionally inactive. Histone tails determines whether the region will be in an actively transcribed state or in an inactive state. Histone modifications can result in alteration of the chromatin structure for transcription machinery or recruiting regulatory proteins. Histones can be modified by methylation, acetylation, ribosylation, ubiquitination, sumoylation, and phosphorylation, which can result in either an increase in transcription or in gene silencing [124]. There are several reports confirming that cancers have altered patterns of histone modifications, chief among them being histone acetylation, histone methylation and histone phosphorylation. Recently, the aberrant histone methylation has been shown to result in cancer-specific loss of expression of surrounding genes. In breast cancer, abnormal histone modification in combination with DNA hypermethylation is frequently associated with epigenetic silencing of tumor suppressor genes and genomic instability [125]. Understanding the mechanisms of dysregulation of histone tail post-translational modifications and their contribution to breast tumorigenesis is critically important in the development of novel targeted therapy for breast cancer patients.

10.4.1 Histone Acetyltransferases

The acetylation of lysine residues on the N-terminus of histones is generally associated with active gene transcription. The HATs can be grouped into three main families based on their sequence similarities: Gcn5/PCAF, p300/CBP and the MYST family of HAT proteins [126]. Most HATs are present as part of large protein complexes and act as transcriptional coactivators. Many of them have also been shown to acetylate proteins other than histones.

10.4.2 Histone Deacetylases

HDACs promote gene repression through removal of acetyl groups from lysine residues in histone tails. At least 18 HDAC genes have been recognized in the

human genome, grouped into three main classes based on sequence homology to the yeast counterparts Rpd3, Hda1 and Sir2/Hst [127, 128]. HDACs act mostly as part of large multiprotein complexes that function as transcriptional corepressors. HDAC family is divided into zinc-dependent enzymes (classes I, IIa, IIb, and IV, of which there are 11 subtype enzymes) and zinc-independent enzymes (class III, also called sirtuins), which require NAD+ for their catalytic activity [129]. Pruitt and colleagues demonstrated that inhibition of class III HDAC SIRT1 using a pharmacologic inhibitor, splitomicin, or siRNA reactivates epigenetically silenced SFRP1, SFRP2, E- cadherin, and CRBP1 genes in human breast cancer cells [130].

HDACs remove the acetyl groups from histone lysine tails and are thought to facilitate transcriptional repression by decreasing the level of histone acetylation. Like HATs, HDACs also have non-histone targets. Several HDACs have been found to be involved in breast cancer. HDAC1 and HDAC4 are overexpressed in breast cancers [131]. In ER-positive MCF-7 cells, expression of HDAC6 was increased after being treated by estradiol, and the elevated HDAC6 could deacetylate alpha-tubulin and increase cell motility [132]. In contrast, ER antagonist tamoxifen (TAM) or ICI 182,780 prevents estradiol-induced HDAC6 upregulation, and then reduces cell motility [133]. Moreover, patients with high levels of HDAC6 tend to be more responsive to endocrine treatment than those with low levels, indicating that the levels of HDAC6 expression might be used both as a marker of endocrine responsiveness and also as a prognostic indicator in breast cancer. Studies have also suggested that sirtuins SIRT3 and SIRT7 are overexpressed in breast cancer [134].

10.4.3 Histone Methyltransferases

Methylation of arginine and lysine residues in histones is involved in the regulation of a wide range of processes including gene activity, chromatin structure and epigenetic memory. Arginine can be either mono- or dimethylated, and in symmetric or asymmetric configurations. Lysine can be in mono-, di- or trimethylated forms. Commonly, lysine methylation at H3K9, H3K27 and H4K20 is associated with gene silencing, whereas methylation at H3K4, H3K36 and H3K79 is associated with gene activation [135].

10.4.3.1 Histone Lysine Methyltransferase (HKMTs)

Histone lysine methylation is a reversible process, dynamically regulated by both lysine methyltransferases and demethylases. Methylation occurs on histone H3 at ε-amino group of lysines 4, 9, 14, 27, 36, and 79 and on histone H4 at lysines 20 and 59 [136]. In general, methylation at H3K4 or H3K36, mono-methylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 are associated with transcriptional activation, whereas trimethylations of H3K27, H3K9 H3K79, and H4K20 are linked to transcriptional repression [137].

Histone methylation is regulated in breast cancer in an even more complicated manner than histone acetylation *via* a large number of chromosomal remodeling regulatory complexes. Modification of H3K4 methylation is catalyzed by the Trithorax group of histone methyltransferases, including SET1 and MLL [138]. The activity of Trithorax proteins is balanced by the opposing effects of the Polycomb group factors, another important histone methyltransferase family that mediates methylation usually associated with epigenetic gene silencing [139].

10.4.3.2 Histone Arginine Methyltransferase (HRMTs)

The protein arginine methyltransferase (PRMT) family is the main HRMTs that act on histones. They are classified into four groups depending on the type of methylarginine they generate: Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4, PRMT6 and PRMT8) catalyze the formation of ω-NG, monomethylarginines (MMA) and NG-asymmetric dimethylarginines (aDMA); Type II PRMTs (PRMT5, PRMT7 and PRMT9) catalyze the formation of MMA and ω-NG, N′G-symmetric dimethylarginines (sDMA); Type III PRMTs catalyze only the monomethylation of arginine residues in proteins; Type IV PRMTs catalyze the methylation at delta (Δ) nitrogen atom of arginine residues [140, 141]. Similar to HKMTs, evidence for the involvement of HRMTs in human cancers is weak. Underexpression of PRMT1 has been reported in breast cancer [142]. PRMT4, also known as coactivator-associated arginine methyltransferase-1 (CARM1), is a coactivator for nuclear receptors and is overexpressed in breast cancers [143]. PRMT4 plays an essential role in estrogen induced cell cycle progression in the MCF-7 breast cancer cells. When stimulated with estrogen, the E2F1 promoter is subjected to PRMT4-dependent dimethylation on H3R17, and this recruitment of PRMT4 by ERα are dependent on the presence of the NCOA3 [144].

10.4.4 Histone Demethylases

It used to be thought that histone methylation was a permanent and irreversible histone modification. However, more recent studies have identified enzymes with the ability to demethylate the methylated histone lysine/arginine residues *via* amine oxidation, hydroxylation or deamination. The histone demethylases could be divided into three distinct classes. The first class (petidylarginine deaminase 4, PADI4) converts a methyl-lysine to citrulline. The second class (lysine-specific demethylase 1, LSD1) reverses histone H3K4 and H3K9 modifications by an oxidative demethylation reaction. The third class of demethylases is the family of Jumonji C (JmjC)-domain containing histone demethylases (JHDMs). In contrast to LSD1, JHDMs can demethylate all three methylated states (mono- di- and tri-methylated lysine). Until now, JHDMs have been found to demethylate H3K36 (JHDM1),

H3K9 (JHDM2A) and H3K9/K27 (JHDM3 and JMJD2A-D) [145]. Histone demethylase JARID1B (PLU-1) is overexpressed in breast cancers but expressed very low in normal adult tissues, and it is essential for the proliferation of the MCF-7 cells and for the nude mice tumor growth of mammary carcinoma cells. Several target genes of JARID1B have also been identified to be associated with proliferation of breast cancer, such as 14–3–3σ, BRCA1, CAV1, and HOXA5 [146]. LSD1 could be a coactivator in the ER signaling. JMJD1C expression is decreased in breast cancer tissues compared with normal breast tissues, suggesting that it might be a tumor suppressor [147].

10.4.5 Histone Phosphorylation in Breast Cancer

Phosphorylation event of histone is thought to have a role in chromatin remodeling and in transcription, and therefore could potentially be associated with the human cancer development. Phosphorylation of H3 on S10 and S28 is essential not only during mitotic chromosome condensation but also in transcriptional activation of immediate early genes. When MCF7 cells were treated with phorbol ester, the number of H3 pS10 foci was increased, and were positioned next to actively transcribed regions in the nucleus. Seemingly, these nuclear sites represent the nuclear location of genes that are induced or in a competent state. Therefore, growth factors stimulating the Ras/MAPK and increasing H3 pS10 at transcriptionally active loci may contribute to aberrant gene expression and breast cancer progression [148].

10.4.6 Other Histone Modifications in Breast Cancer

Apart from acetylation, methylation and phosphorylation, there are some other modifications of histones occur. These epigenetic changes include ubiquitination/sumoylation, ADP-ribosylation, deamination, and proline isomerization. However, their function and mechanism is not clear, some studies have showed that they are also associated with breast cancer and other human cancers.

Regulation of the expression of genes by phosphorylated and undersumoylated PRs is a novel form of hormone independent PR action that is predicted to contribute to breast cancer cell growth and survival [149]. Recently, E3 ubiquitin ligase has been shown to play important role in breast carcinogenesis. Ubiquitin-mediated protein degradation plays an important role in many cancer-related cellular processes. E3s play crucial role because they control the specificity of the substrate. Gathering evidence suggests that genetic and expression alteration of E3s plays important role in breast carcinogenesis [150]. Sumoylation of histone also seem to govern chromatin structure and function to mediate transcriptional repression and gene silencing [151].

10.5 Polycomb Group (PcG) Proteins

The Polycomb gene was discovered about 60 years ago as a mutation inducing a particular homeotic phenotype. Later studies indicated that Polycomb is a general repressor of homeotic genes [152]. Genes with similar functions were identified and grouped under the name Polycomb group (PcG) genes. These genes have demonstrated epigenetic regulation of genes during development and differentiation. These proteins function to maintain a silenced state of developmental regulators. The PcG genes encode subunits, which are part of the polycomb repressive complex. Furthermore, these PcG genes are closely associated with coordinated regulation of histone modification and methylation, thus inter connecting the various epigenetic mechanisms. The Polycomb Repressive Complex 1 (PRC1) plays a crucial role in reading histone methylation marks and silencing target genes [153]. PRC1 can be recruited to chromatin by the PC chromodomain-mediated recognition of the H3K27me3 mark, which is deposited by PRC2. PRC1 components can also repress transcription without directly contacting transcription factors. Isolation of a core PRC1 complex, called PCC, that contains PC, PH, PSC and dRING1, revealed that these four PcG proteins are sufficient to inhibit ATP-dependent chromatin remodeling by the SWI/SNF complex, a homolog of the Drosophila BRM complex.

The Polycomb group (PcG) comprises several proteins that form multiprotein complexes, 2–5 MDa in size, that regulate gene activity at the chromatin level. The first components of Drosophila PcG were identified in 1980s and after several years it was recognized in mammalian cells, indicating strong evolutionary conservation [154]. PcG proteins, along with the counteracting Trithorax group (TrxG) proteins, were initially recognized as part of the memory system that transmission of cell identities throughout cell division [155]. PcG protein expression appears to be tightly regulated during normal cell proliferation and differentiation. While the expression, of PcG is frequently dysregulated in several cancer types [156]. Several PcG genes regulate self-renewal of specific stem cells, suggesting a link between the maintenance of cell homeostasis and carcinogenesis [157]. Bmi-1 was initially identified as an oncogene that cooperated with c-Myc in the generation of mouse pre-B-cell lymphomas [158]. It is also considered to be the first functional mammalian PcG protooncogene, and has been implicated in axial patterning, hematopoiesis, cell cycle regulation, and senescence. Data obtained in mice and *in vitro* studies have indicated that Bmi-1 protein regulates the INK4a/ARF locus, which encodes two unrelated tumor suppressors, p16INK4a and p19ARF (p14ARF in humans), which act in the two main cell cycle control pathways (pRb and p53, respectively) [159]. However, the effect of Bmi-1 overexpression on the inactivation of the INK4a/ARF transcripts in human tumorigenesis is unclear. One study demonstrated that although high levels of Bmi-1 were frequently observed in tumors, they did not correlate with downregulation of p16INK4a or p14ARF. A correlation between c-Myc and Bmi-1 expression levels has been shown; however, tumors showing elevated expression of both genes were not associated to a worse prognosis.

10.6 Non-coding RNAs

Many non-coding RNAs such as microRNA, small nucleolar RNAs, and repeat-associated small interfering RNA have been shown to alter transcription. MicroRNAs are small non-coding RNAs of approximately 22 nucleotides [160]. Several genes that are responsible for breast cancer progression are shown to be targeted by microRNAs, for example, miR-199b-5p was shown to inhibit HER2 expression by directly targeting its 3′-untranslated region (3′UTR) in breast cancer cells [161].

10.6.1 MicroRNAs Inhibit Target mRNA Translation

Initial studies on miRNAs suggested that these small non-coding RNAs inhibit mRNA translation, with perfect or near perfect complimentarity inducing mRNA degradation, while imperfect binding resulting in inhibition of translation [162]. Oncogenic microRNAs are also called as OncomiRs and they have been shown to promote breast cancer. These include miR-10 family, which regulate Hox transcripts [163]. In case of breast cancer, miR-10 family is reported to be involved both in the development and metastasis through miR-10a and miR-10b, respectively. Expression of miR-21 is negatively correlated with expression of PTEN in breast cancer and also correlates with advanced stage and metastasis and poor survival [164]. Finally, miR-17 ~ 92 cluster is a polycistron and is located in a region of DNA that is amplified in various cancer. The expression of miR-17–5p is increased in invasive MDA-MB-231 cells but not in non-invasive MCF-7 breast cancer cells [165]. Ectopic expression of this miRNA in MCF-7 cells can lead to more invasive and migratory phenotypes by targeting HBP1/β-catenin pathway. Similarly, down regulation of miR-17–5p suppresses the migration and invasion of MDA-MB-231 cells in vitro.

There are also tumor suppressor microRNAs. The Let-7 family includes members that affect muscle formation, cell adhesion and regulation of gene expression and development [166]. Let-7 expression is lost in breast cancer at an early stage of disease progression in breast cancer. Similarly, the miR-200 family is lost in invasive breast cancer cell lines with mesenchymal phenotypes and also in regions of metaplastic breast cancer specimens lacking E-cadherin. The miR-200 family has been shown to regulate PLCG1, Bmi1, TGF-β2, FAP-1, ZEB and Suz12, hence acting as tumors suppressor [167, 168]. The expression of miR-205 is restricted to basal epithelium of normal mammary ducts and lobules, but its expression is reduced or lost in tumor [169]. Ectopic expression of miR-205 in breast cancer cells inhibits invasion, proliferation and anchorage independent growth, in part through direct targeting of Her3 and VEGF-A [170]. In addition, miR-145, is significantly down-regulated in breast cancer specimen compared with normal breast tissue [171]. Furthermore, miR-145 can directly target estrogen receptor-α (ER-α) protein expression through direct interaction and promotes apoptosis in both ER-α positive and wild type TP53-expressing breast cancer cells.

Breast cancer metastatic related microRNAs, there are miRNAs that promote the metastasis in breast cancer include miR-9, miR-10b, miR-21, miR-29a, miR-155 and miR-373/520 family. On the other hand there are microRNAs reported to suppress metastasis and they are, miR-7, miR-17/20, miR-22, miR-30, miR-31, miR-126, miR-145, miR-146, miR-193b, miR-205, miR-206, miR-335, miR-448, miR-661 and let-7 [172].

10.6.2 MicroRNA Control of Epigenetic Mechanisms

Both miRNAs, and small interfering RNAs (siRNAs) are involved in both DNA methylation and histone modifications. Maison et al. showed that RNAse treatment can abolish the localization of methylated H3 lysine 9 and HP1 to pericentromeric chromatin [173]. Fukagawa et al. demonstrated that Dicer-related RNAi machinery is necessary for the formation of heterochromatin structure [174]. Furthermore, miR22, miR206, and miR-221/222 regulate ER-α expression in breast cancer [175]. MicroRNAs can target genes coding for enzymes responsible for histone modification (EZH2) and DNA methylation (DNMT3A and DNMT3B) [176]. miR-101 and miR-26a target the EZH2 mRNA 3′UTR and inhibits its translation [177].

Studies from Shimono et al. reported that 37 miRNAs are differentially expressed in CD44+/CD24-/low breast cancer stem cells as compared with non-tumorigenic cancer cells. In particular, three clusters, miR-200c-141, miR-200b-200a-429 and miR-183–96–182, are significantly down regulated. Furthermore, loss of p53 leads to a decreased level of miR-200c and an increase in the expression of EMT and stemness markers, leading to the development of a high tumor grade [178]. In addition, Han and colleagues isolated ALDH1+ and CD44+/CD24-/low cells from MCF-7 parental cells and found that HIF-1α and miR-21 are upregulated in the stem-like cells [179]. Moreover, reduction in miR-21 expression by antagomir leads to reversal of EMT, downregulation of HIF-1α, as well as suppression of invasion and migration. This indicates that miR-21 regulates EMT transition in breast cancer stem cells as well as HIF-1α overexpression.

10.6.3 Epigenetic Control of MicroRNA Expression

While microRNAs regulate epigenetic mechanisms, microRNA expression itself can be regulated in a similar manner. Interestingly, CpG island methylations in miRNA regions influence miRNA function, thereby altering the processes of tumorigenesis [180]. Nearly half of all identified miRNAs are associated with CpG sites, and studies revealed methylation levels at several miRNA loci across normal and malignant cell lines [181]. Silencing of miRNA gene expression due to hypermethylation is also a feature of several cancers. MicroRNAs can trigger transcriptional silencing *via* chromatin remodeling [182]. Tumor suppressor miRNAs could undergo aberrant DNA methylation accompanied by histone modifications associated

with transcriptional inactivation. Interestingly, miR-124a silencing by DNA methylation was accompanied by the absence of active histone markers, such as acetylation of histone H3, acetylation of histone H4, trimethylation of histone H3-lysine 4, and occupancy by MBDs such as MeCP2 and MBD2 [183]. The epigenetic regulation of miR-196a-2 in breast cancer development has also been studied. Hypermethylation of a CpG island 700 bp upstream of the miR-196a-2 precursor promoter was associated with reduced breast cancer risk [181]. Putative targets for the microRNA include HOXD10, LSP1 and TOX3. HOXD10 is a target for initiation of breast cancer invasion and metastasis, *LSP1* and *TOX3* (TNRC9) were identified as novel breast cancer susceptibility markers in large-scale whole-genome association studies [184]. Furthermore, miR335 locus on 7q32.2 is a selective metastasis suppressor and tumor initiation suppressor locus in human breast cancer. In fact, miR-335 regulates a set of genes that regulate metastasis [185]. This locus also undergoes epigenetic hypermethylation in every patient metastatic cell population. In addition, genetic deletion of miR-335 is a common event in human breast cancer [185]. There are several other miRNAs that are regulated by hypermethylation of the promoter region including miR-9-1 (targets transcription factor REST and its partner CoREST), miR-124a3, miR-148, and miR663 [186].

10.7 Epigenetics and Cancer Stem Cells

Cancer stem cells (CDCs) are relatively rare subpopulation of cells within a tumor that has the ability to initiate new tumor growth and have the capacity to self-renewal, the use of key regulatory pathways and establishment of dynamic epigenetic profiles. Compared to normal adult stem cells, CSCs will have higher proliferative rates and less dependence to stem cell niche. They may give rise to cellular heterogeneity in the tumor by initiating epigenetic reprogramming, this is because most of the currently known markers including CD44, Bmi1, ALDH1, CD133, and EPCAM have been shown to be regulated by either DNA methylation or histone modifications [187]. Furthermore, analysis of DNA methylation profile of CSCs, suggest that epigenetic markers of stemness of CSCs resemble embryonic stem cells rather than the adult normal stem cells pattern. The major problem with cancer chemotherapy is the development of resistance to the drugs, especially in CSCs; therefore understanding the epigenetic profile of these CSCs will provide us new avenues for developing new therapeutics for breast cancer.

10.8 Epigenetic Modifier Drugs Against Breast Cancer

There are many drugs in clinical trials for breast cancer that are epigenetic modifiers (Table 10.2), most of which come under the classification of HDAC inhibitors. Vorinostat a hydroxamic acid is being used as a single agent or in combination with tamoxifen, aromatase inhibitor, paclitaxel, carboplatin, trastuzumab, ixabepilone,

Table 10.2 Epigenetic drugs in clinical trials

Classification	Drugs in Pre-clinical studies	Drugs in clinical trial
DNMT inhibitors		
Nucleoside analogues	RG108, MG98, NVP-LAQ824, Decitabine, SGI-110	5-azacytidine, 5-aza-2′-deoxycytidine, 5′-fluro-2′-deoxycytidine, Zebularine, Epigallocatechin-3 gallate, Hydrazine
HDAC inhibitors		
Short chain fatty acids		Valproic acid and phenylbutyrate
Hydroxamic acids		Suberoylanilide hydroxamic acid (SAHA), Panobinostat, Belinostat, CHR-3996, Tefinostat, JNJ-26481585
Cyclic tetrapeptides	Trapoxin, Despsipeptide, cyclic hydroxamic acid-containing peptide (CHAP), and Apicidin	
Benzamides		Entinostat (MS-275), N-acetly-dinaline (CI-994), Mocetinostat (MGCD-0103)

lapatinib [188]. Another HDAC inhibitor in the breast cancer clinical trial is Entinostat, which comes under Benzamide sub class, this agent is being combined with exemestane, Anastrozole and lapatinib [188].

10.8.1 Nucleoside Analogs

There are several nucleoside analogue methylation inhibitors such as 5-azacytidine, 5-aza-2′-deoxycytidine, 5′-fluro-2′-deoxycytidine and Zebularine. These inhibitors affect DNA synthesis and are implicated to regulate DNA methylation. The formation of covalent complexes with DNMTs results in depletion of the enzyme and finally, a reversal of the methylation pattern. The non-nucleoside analogues are shown to inhibit DNA methylation by binding directly to the catalytic region of the DNMT without incorporating into DNA. RG108, was shown to inhibit DNA methyltransferase [189], however, it has not entered clinical trials. A non-toxic antisense oligonucleotide, MG98 has been shown to prevent translation of DNMT1 mRNA by hybridizing to the 3′UTR of the DNMT1 mRNA [190]. In addition, NVP-LAQ824, a derivative of a compound derived from the sponge *Psseudoceratina purpurea* called Psammapalin, inhibits both DNMTs and HDACs [191]. Furthermore, there are several other agents such as Decitabine and SGI-110 that are nucleoside analogues that inhibit DNMT [188, 192]. The green tea compound epigallocatechin-3 gallate (EGCG) has also been shown to reduce DNA methylation and increase transcription of tumor suppressor genes [193]. Currently, EGCG is being tested in clinical trial [194].

10.8.2 Histone Deacetylation Inhibitors

These are agents that inhibit histone deacetylase enzymes leading to increased acetylation in histones and resulting in altered cellular processes that have become defective in cancerous cells. The compounds are divided into short chain fatty acids, hydroxamic acids, cyclic tetrapeptides and benzamides. Currently, there are several HDAC inhibitors in clinical development for treatment of variety of cancers.

10.8.3 Short Chain Fatty Acids (SCFA)

HDAC inhibition by SCFAs was first demonstrated using butyrate resulting in hyperacetylate of histones H3 and H4 [195]. Valproic acid and phenylbutyrate is now used clinically as histone deacetylase inhibitor for breast cancer [196]. They induce proteosomal degradation of HDAC2 by inhibiting the catalytic activity of class I HDACs.

10.8.4 Hydroxamic Acids

Trichostatin A is an HDAC inhibitor that can inhibit the viability of breast cancer cells [197]. Suberoylanilide hydroxamic acid (SAHA) is another molecule that has been shown to inhibit both class I and II HDAC enzymes [198]. SAHA has FDA approval for treating cutaneous manifestations of lymphoma patients. Furthermore, there are other drugs such as Panobinostat, Belinostat, CHR-3996, Tefinostat, JNJ-26481585 that have entered the clinical studies [188].

10.8.5 Cyclic Tetrapeptides

Trapoxin accumulates highly acetylated core histones [199]. Even low concentrations of trapoxin can bind to histone deacetylase through epoxide moiety and inhibit deacetylation of acetylated histones [200]. Despsipeptide, a bicyclic peptide increases p53 expression in breast cancer cells [201]. In addition, CHAP, cyclic hydroxamic acid-containing peptide has been shown to inhibit HDAC [202]. Apicidin, a cyclic tetrapeptide increases the levels of acetylated histone H3 and H4 in breast cancer cells [203].

10.8.6 Benzamides

There are many benzamides that are either in clinical trials or are in preclinical testing. Entinostat (MS-275) treatment results in hyperacetylation of nuclear histones in

various tumor cells [204]. This class I HDAC selective inhibitor is currently being used in the phase II clinical trials for triple negative breast cancers. In addition, Mocetinostat (MGCD-0103), a class-selective HDAC inhibitor with IC50s in the submicromolar range has entered clinical trials, although not for breast cancers [188]. Finally, N-acetyldinaline (CI-994), another histone deacetylase inhibitor with a substituted benzamide derivative causes accumulation of acetylated histones [205]. The combination of current therapy with these novel inhibitors can result in successful treatment of breast cancer.

10.9 Conclusion

Over the last decade our knowledge on hypermethylation of DNA sequences and histone modifications along with Polycomb group proteins and microRNAs, and their epigenetic role in regulation of breast cancer gene expression has been increased tremendously. However, much needs to be done to understand the exact mechanisms initiating these changes during tumor development and progression. There may be a critical cross talk between the genetics and epigenetics, which has to be studied thoroughly to enhance our understanding of breast cancer initiation and progression. Identifying the epigenetic signature of breast cancer and the molecular mechanisms will definitely enhance the treatment opportunity.

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Chapter 11
Exploiting Epigenetic Modifiers to Circumvent Melanoma Dual Resistance to TCR-Engineered Immunotherapy- and $BRAF^{V600E}$-Kinase Inhibitor

Ali R. Jazirehi

Abstract The discovery of activating $BRAF^{V600E}$ mutation in vast majority of melanoma patients has paved the way for novel drug discovery. Targeted therapy using selective $BRAF^{V600E}$ inhibitor Vemurafenib (PLX4032) and adoptive cell therapy (ACT) using MART-1 T-cell receptor (TCR)-engineered T lymphocytes (F5 CTL) both produce dramatic, but transient, clinical responses in most patients with metastatic melanoma. Adoption of bypass survival signaling pathways (e.g., AKT) and aberrant apoptotic machinery may confer resistance to death signals delivered by Vemurafenib and transgenic CTLs. We have established an *in vitro* model of resistant (R) lines from F5 CTL- and Vemurafenib-sensitive lines harboring $BRAF^{V600E}$ under selective pressure. Interestingly, PLX-resistant tumors, while surviving high PLX4032 concentrations, develop cross-resistance to F5 CTL-killing, suggesting the use of a common apoptotic pathway by both modalities. Preliminary experiments suggest that the acquired resistance can be reversed with the histone deacetylase inhibitor (HDACi) SAHA, possibly through modulation of the expression profile of apoptotic genes. Future studies are warranted to identify the bypass signaling pathways and the molecular determinants responsible for immune- and PLX-resistance. Moreover, the exact underlying molecular mechanisms of SAHA-mediated immunosensitization need to be defined. However, these and other studies suggest that the addition of an HDACi to $BRAF^{V600E}$-based targeted therapy will immuno-sensitize PLX-resistant metastatic melanomas to F5 CTL ACT.

Keywords Histone deacetylase inhibitor • Gene regulation • Apoptosis • Melanoma immunotherapy • Vemurafenib • BRAF • Targeted therapy • Sensitization

A.R. Jazirehi, CLS, Ph.D. (✉)
Division of Surgical Oncology, Department of Surgery, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine at UCLA, University of California, Los Angeles, 10833 Le Conte Avenue, Los Angeles, CA 90095-1782, USA
e-mail: ajazirehi@mednet.ucla.edu

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11.1 Introduction

The main function of DNA, namely to carry genetic information, was first revealed in 1944 [1], which elicited further investigations of the DNA structure, which culminated in Watson and Crick's model [2]. In the double helix model, it was proposed that DNA consists of two strands made of simple repeating units called nucleotides; each strand is made of a sugar-phosphate backbone, with one of four nitrogenous bases (adenine: A, thymine: T, guanine: G, cytosine: C) attached to each sugar. Each backbone has a 5′ to 3′ directionality, with an overall anti-parallel orientation of the two strands. Furthermore, each base is paired with the complementing base on the other strand: G with C, and A with T. Soon after its discovery, the general belief was that DNA is solely responsible for generating the full spectrum of information that eventually give rise to a complex organism like a human. The current view, however, entails that epigenetics govern the mechanisms responsible for DNA storage and recovery. The term "epigenetic" refers to heritable changes affecting gene expression carried out by means other than changes in the primary nucleotide sequence. DNA methylation and covalent modifications of histone proteins are examples of epigenetic alterations that have been substantially studied [3]. The disturbance of the intricate epigenetic balance by such alterations can lead to chromatin remodeling, with significant impact on gene transcription. Alterations in DNA methylation patterns resulting in DNA hyper- and/or hypomethylation alter gene expression profile frequently seen in cancer. Inside all eukaryotic nuclei, DNA is highly folded and compacted by a series of histone and non-histone proteins; this mixture is called the chromatin. The basic repeating unit of chromatin is the nucleosome; 146 base pairs of DNA wrapped around an octamer of core histones. Certain epigenetic alterations occur through covalent modification of histone amino (N)-terminal tails. The collection of these modifications allow for a much greater information capacity in the DNA [4].

Histones guide the interactions between DNA and other proteins, such as transcription factors. A total of six classes of histones exist, which can be organized into two main classes: core histones (H2A, H2B, H3, and H4) and linker histones (H1 and H5). Two copies of each of the core histones are assembled together to form an octameric histone core. This octamer along with approximately 146 base pairs of DNA wound around the core forms a nucleosome (measuring approximately 10 nm in diameter), the basic structural subunit of chromatin. The linker histone H1 binds the nucleosome at the entry and exit sites of the DNA and as a result can lock the DNA into place. The histone octamer can be dissociated into an $(H3–H4)_2$ tetramer and two H2A-H2B dimers [5].

Gene expression is a three step process that proceeds in this order: transcription, translation, and post-translational modification(s) of a protein. The central dogma of gene expression states that DNA is transcribed into mRNA and subsequently translated into a functional protein. To initiate transcription RNA polymerase II needs to bind the promoter sequence, which will then recruit additional regulatory factors collectively called general transcription machinery. In eukaryotes the process of transcription is mediated by a group of proteins called transcription factors.

As transcriptions proceeds, RNA polymerase II transcribes an mRNA strand that is complementary to the template DNA strand. The transcribed mRNA strands are then translated into specific amino acid sequences that combine to form a protein [6]; the functional unit of a living cell.

11.2 Post Translational Histone Modifications and Their Role in Gene Expression

Oftentimes, almost immediately after or even during protein synthesis, the residues in a protein are chemically modified, which lead to changes in the physical and chemical properties as well as the stability, folding, activity, and, function of the protein. Post-translational modifications include addition of functional groups such as acetyl, methyl, and phosphate groups and SUMOylation, which involves covalent attachment and detachment of SUMO (Small Ubiquitin-like Modifier) proteins; changing the chemical nature of an amino acid, and making structural changes in the protein [7].

Histone N-terminal tails are subject to a diverse array of post-translational modifications. Of the histone core, lysine (Lys; K) residues in the highly conserved N-termini of histones H3 and H4 have a tendency to undergo chemical modifications such as acetylation and methylation [5]. These modifications not only cause changes in chromatin structure, but also affect the dynamics of regulatory factors. In acetylation, a process in which acetyl groups are introduced into a compound and later replace an active hydrogen atom, histones are acetylated on lysine residues in the amino-terminal tail. This process culminates in neutralizing the positively charged lysine residue and, hence, decreasing its interaction with the negatively charged phosphate groups of the DNA backbone. Subsequently, chromatin switches from a condensed form (heterochromatin) into a more relaxed configuration, which is no longer bound to a histone octamer. Therefore, acetylation results in activation of gene transcription. Different components of the transcriptional machinery, such as various transcription factors and RNA polymerase II, are then able to bind to the promoter sequence, with replication and transcription ensuing. Acetyl groups that bind to the nucleosomal units could affect transcription in two ways—they either act as physical barriers, preventing transcriptional repressors from binding to the nucleosome, or they function as docking sites for other proteins such as transcriptional activators. Both mechanisms facilitate gene transcription. Specific examples of residues that are subject to acetylation include H3K9, H3, K18, and H4K12 [8].

However, in methylation a repressed chromatin structure tightly holds DNA together so that it is inaccessible to transcriptional machinery. Genes are silenced through DNA methylation, as the enzymatic addition of methyl groups to cytosine nucleotides in DNA occur. Although, methylation is usually associated with transcriptional repression, the effect of this modification actually depends on the particular lysine or arginine (R) residue involved. For instance, transcriptional repression is observed in the methylation of H3K9 in heterochromatin and repressed genes, whereas transcriptional activation is observed in the methylation of H3K4 in

euchromatin and active genes [5, 8]. Methylation of H3K27 is another epigenetic mark for transcriptional repression. In addition to the various histone H3 lysine-residue methylations, histone H4 can also undergo methylation at lysine 20 (H4K20) resulting in a silent chromatin structure [8]. Although R residues, such as demethylated H4R3, can only be methylated once or twice by peptidylarginine methyltransferases, K residues can be methylated once, twice, or three times by lysine methyltransferases, resulting in three distinct H3K9 methylation states: mono-, di-, and tri-methylated states—denoted as H3K9me1, H3K9me2, and H3K9me3, respectively [8].

Further expansion on methylation and its role in repressing chromatin leads to the discussion of CpG island methylation, which also corresponds to heterochromatic regions. A CpG island is a region of a genome characterized by a high frequency of C-G nucleotides united through a phosphodiester bond. These 200 base pair islands are normally located near or in the promoter regions of mammalian genes. Methylation of CpG sites in these promoter regions may result in gene repression. Furthermore, regions of specialized chromatin are produced when factors such as CpG methylation, the proteins that attach to them, and repressive histone modifications coalesce together, resulting in transcriptional repression. In the case of cancer, this observed gene silencing disables critical proteins, such as tumor suppressors PTEN and p53 [5, 8]. Another post-translational modification is phosphorylation, which involves the addition of a phosphate group to a protein by a kinase. Phosphorylation plays a critical role in an extensive array of cellular processes such as activation of signal transduction pathways. Dephosphorylation, on the other hand, is mediated by the enzyme phosphatase, which removes phosphate groups. Phosphorylation and dephosphorylation result in turning various enzymes and receptors “on” and “off”. In eukaryotic proteins, phosphorylation usually occurs on serine (Ser, S), tyrosine (Tyr, Y), and threonine (Thr, T) residues. The addition of a phosphate molecule to the polar R groups of these amino acids can cause a conformational change in the protein by changing a protein’s hydrophobic portion into one that is hydrophilic [6]. Lastly, SUMOylation, functions in protein stability, apoptosis, nuclear cytosolic transport, and transcriptional regulation. It involves the addition of one or more copies of the 101-amino-acid polypeptide SUMO (small ubiquitin-like modifier) to a protein’s lysine residues. SUMOylation activity is analogous to ubiquitination, but the results are different. SUMOylation has been associated in a variety of cancers, signifying that its manipulation could be one potential method for regulating cancer development. A recent study implicates SUMOylation as a potential regulator of the NF-κB signaling pathway. NF-κB is involved in a myriad of cellular responses, especially those of the immune system: from lymphocytes differentiation to the fine tuning of immune responses. Activation of NF-κB is involved in the stimulation of the adaptive, specific, immune response. Since the adaptive response is largely responsible for anti-tumor effects and malignant cell cytotoxicity, attention has been turned towards specific modulation of the NF-κB pathway.

In addition to the gene silencing processes observed in DNA methylation, the processes of histone deacetylation, in which acetyl groups are removed from lysine residues of histone tails, also correspond to transcriptionally silenced chromatin.

In contrast to the nucleosomal relaxation caused by histone acetyl-transferases (HAT); enzymes that catalyze the addition of acetyl groups to histone N-terminal lysine residues, histone deacetylases (HDACs) increase the ability of histones to bind to DNA, thus, promoting DNA condensation and inhibiting the chromatin expansion, which prevents gene transcription. Histone acetylation plays a vital role in the regulation of gene expression, and the epigenetic mechanism (reversibly altering the terminal tails of core histones which remodels the higher order chromatin structure and therefore controls genetic expression) displayed in HDAC activity may be brought to a standstill with the discovery of HDAC inhibitors (HDACis) [9]. HDACs are classified into four classes based on their homology to yeast histone deacetylases. Class I includes HDAC1, -2, -3, and -8 (which are related to the yeast RPD3 gene) and Class II includes HDAC4, -5, -6, -7, -9, and -10 (which are related to the yeast Hda1 gene). The two remaining classes include the sirtuins 1–7 that belong to Class III, and HDAC11, which has features of both of the first two classes, and belongs to Class IV [10]. In summary, class I, II and IV HDACs which are zinc-dependent deacetylases could be impeded by several HDACis including SAHA, TSA, and LBH589 [10]. However, class III HDACs which are NAD^+-dependent could not be impeded by hydroxamic acid-based HDAC inhibitors.

The activation of cancer-promoting oncogenes, which protect against apoptosis, results in uncontrolled growth and division in cancer cells. This aberration inactivates tumor suppressor genes that promote apoptosis and regulate cell cycle activities leading to mistakes in DNA replication and cell cycle. Studies indicate that several tumor types such as Cutaneous T-Cell Lymphoma (CTCL), Acute (AML) and Chronic Myelogenous Leukemia (CML), breast, colon, and prostate cancer exhibit an overexpression of HDACs along with a disruption in HAT activity, leading to a hypoacetylated chromatin structure. The hypoacetylated chromatin could lead to a decreased expression of pro-apoptotic (e.g., Bax, Bak, Bid, Bim, etc.) or tumor suppressor genes (e.g., Retinoblastoma protein (pRb), p53, and PTEN) [9]. HDACis can stimulate apoptosis, induce cell cycle arrest, promote protein-DNA interactions leading to cellular differentiation, inhibit migration, invasion, and angiogenesis in various tumor models [9].

11.3 Suberoylonilide Hydroxamic Acid SAHA in Cancer Therapy

The orally administered HDACi SAHA (Vorinostat, Zolinza®) is the only Food and Drug Administration (FDA) approved HDACi used for the treatment of cutaneous T-cell lymphoma (CTCL) [11]. Since its discovery as an HDACi, the effects of SAHA have been tested on different cell types including: CTCL, pituitary adenomas, cervical cancer, oral squamous cell carcinoma (OSCC), prostate cancer, mesenchymal stem cells, neuroblastoma, retinoblastoma, effector and regulatory T-cells, acute lymphatic leukemia, hepatoma, breast cancer, mantle cell lymphoma, and liver cancer [12, and references therein]. Pituitary adenoma accounts for 15–20 %

of intracranial tumors. Although radiotherapy and surgery continue to serve as main treatment options, treatment of this invasive tumor requires novel therapeutics. A recent study indicated that treatment of the GH3 pituitary adenoma cells with 500 nM-4 μM SAHA induces growth arrest and promotes cell cytotoxicity that are induced by poly ADP-ribose polymerase (PARP) cleavage and procaspase-3 activation. In addition, SAHA down-regulates XIAP, survivin, Bcl-2, and Bcl-$_{xL}$ but there was no change in Bax expression [13]. Results of another study indicate a dose-dependent suppression of the *in vitro* growth of OSCC cells treated with SAHA due to cell cycle arrest at the G1 phase as well as a reduction in the percentage of S-phase cells. Also hyperacetylation of p53 subsequent to treatment with SAHA (0.7–1.7 μM) was observed. These findings propose that treatment of OSCC with SAHA blocks the growth of the tumor in the G1 phase. A current study showed the potential of HDACis in treating retinoblastoma in transgenically rb/rb mice. The pro-apoptotic effects of SAHA and two other HDACis, TSA, and MS-275, were analyzed by caspases-3, -7 activity, Annexin V translocation, and Bim expression levels. All three HDACis promoted apoptosis in a dose-dependent manner and the animals demonstrated reduced RB cell survival and decreased tumor burden highlighting the potential of using HDACi in retinoblastoma. Despite having relatively low toxicity on normal cells, SAHA is suspected to have some adverse effects on the bone marrow and pleuripotency of stem/progenitor cells. The bone marrow microenvironment contains several types of stem cells, including mesenchymal stem cells (MSCs). Experimentally, treating MSCs with SAHA exhibits cell cycle arrest and apoptosis. Using human hepatoma HepG2 cells, SAHA (10 μM) induced apoptotic effects after a lag phase of 12–16 h, and more than 80 % of the SAHA treated cells were in G0-G1 phase. Noteworthy, while inducing apoptosis, SAHA also activates NF-κB. The NF-κB pathway is often activated in cancer cells, and contributes to their survival and proliferation. The results of one study suggest that simultaneous treatment of the cells with the proteasome inhibitor MG-132 and SAHA dramatically reduces enhanced NF-κB activity. Another study further investigated the effects of combination of SAHA and Bortezomib. The results showed synergistic apoptosis and higher levels of caspases-3, -8, and -9 along with cytoplasmic accumulation of IκBα resulting in lower NF-κB activity. Moreover, SAHA induces the re-expression of DR5 in TRAIL-insensitive malignant cells and subsequently the cells become sensitive to TRAIL-mediated apoptosis [12–14].

Aside from its anti-tumor benefits SAHA can down-modulate the immune system by up-regulating the $CD4^+CD25^+Foxp3^+$ T-regulatory (Treg) cells while having an anti-proliferative effect on effector T cells resulting in dampening the immune response. Thus, SAHA can be used as a means of immuno-suppression to allow for increased chances of tolerance in graft transplantation [12 and references therein].

Altogether, numerous clinical and pre-clinical investigations have verified that SAHA is capable of introducing cell cycle arrest and triggering apoptotic pathways. Therefore, with the ongoing research for a cure for cancer, HDACis have surely presented themselves as a promising subgroup of compounds that function in epigenetic therapy.

11.4 Melanoma: A Rapidly Growing Global Health Threat

Melanoma is an aggressive form of skin cancer that originates in melanocytes, specialized pigment-producing cells found predominantly in the basal layer of the epidermis and the eyes. Normally, the melanocytes synthesize melanins. When skin is exposed to ultraviolet (UV) radiation, melanocytes are stimulated by epidermal keratinocytes to increase melanin production, causing the skin to tan. Hence, melanocytes play a key role in protecting the skin from the damaging effects of UV radiation and in preventing skin cancer [15]. Consequently, people with fair skin or those who lack functional melanocytes in pigmentary disorders such as vitiligo and albinism are at a greater risk to UV radiation [16].

Skin cancer is the most common human malignancy in the United States where nearly one in five Americans will develop it in their lifetime [17]. Melanoma is the least common of the skin cancers with basal cell carcinoma and squamous cell carcinoma making up the vast majority of cases. According to the American Cancer Society, melanoma constitutes only 3 % of all cases, yet it is responsible for more than 75 % of skin cancer-related deaths. More than 20 Americans die every day from skin cancer, primarily from melanoma, amounting to one death nearly every hour (~62 min). Looking at the number of cases and deaths last year in the United States, the National Cancer Institute (NCI) estimates 68,720 new cases were diagnosed with 8,650 deaths.

Melanoma may develop in people of all ages, ethnic groups and sexes, though the rate of incidence is distinctly higher in particular groups. According to the NCI, the majority of people diagnosed with melanoma are Caucasian males over age 50. Until age 39, women are twice as likely to develop melanoma as men; but at ages 40 and over, incidence in men exceeds women, becoming more pronounced with each decade [18]. In addition, skin tone appears to play a major role in melanoma incidence, as populations with darker skin such as African Americans, Latinos and Asians have a lower rate of incidence than Caucasians. However, melanoma is frequently fatal for these groups. Furthermore, among non-Caucasians, melanoma is a higher risk for children than adults, where 6.5 % of pediatric melanomas occur in non-Caucasians [19].

11.5 Treatment Options for Melanoma

Despite impressive numbers of ongoing clinical trials, the success of systemic therapy of metastatic melanoma has been minimal at best. Depending on how far melanoma has advanced, the success of therapeutic strategies will vary greatly. If melanoma is diagnosed at the very early stages before the malignant melanocytes become invasive, surgical resection of the primary tumor is extremely promising with over a 95 % success rate [18]. Unfortunately, melanoma lesions are commonly asymptomatic for extended periods of time. Upon acquisition of metastatic potential,

surgical excision is rendered useless and the condition becomes generally fatal. Chemotherapeutic regimens utilizing a single agent (Dacarbazine and Temozolomide), combination chemotherapy (cisplatin, vinblastine, DTIC; CVD) and combination of chemotherapy and immunomodulatory agents (Tamoxifen and interferon-α (IFN-α)) have demonstrated modest results. Single agent DTIC is currently the only FDA approved drug but has produced response rates in only 15–20 % of patients with a median survival rate of 6 months and a 5 year survival rate of <5 % [20, 21]. Combination chemotherapies have been evaluated in an attempt to improve response rates utilizing two to three drug regimens with or without tamoxifen. However, most trials did not consistently demonstrate an improvement in response rates or overall survival compared to the single-agent DTIC [18–21].

11.6 Adoptive Cell Therapy (ACT) for the Treatment of Metastatic Melanoma

In recent years, there has been growing enthusiasm to harness the immune system for cancer therapy. Initial attempts at immunotherapeutic strategies demonstrated promising results for its time with response rates in the order of 5–15 %, though these responses were frequently durable and resulted in clinical benefit in a subset of patients [22, 23]. Interferon-α (IFN-α) was the first exogenous cytokine to demonstrate antitumor activity against advanced melanoma. IFN-α2b is a highly pleiotropic cytokine with immunoregulatory, antiproliferative and antiangiogenic properties in multiple malignancies, leading to its approval for adjuvant treatment of stage Iib/III melanoma by the Food and Drug Administration (FDA) in 1995. It remains the only approved adjuvant therapy for patients with high risk for recurrence and death, though tolerability is an issue due to flu-like symptoms, fatigue, anorexia and depression [24].

The T-cell growth factor IL-2 was the second promising exogenous cytokine to demonstrate antitumor activity, received FAD approval in 1998 for treatment of adults with advanced metastatic melanoma. IL-2 is a key player in immune regulation and T-cell proliferation. High-dose bolus intravenous IL-2 was shown to activate endogenous tumor-reactive cells *in vivo* and caused regression of some human solid tumors. Retrospective long-term analysis of phase II studies demonstrated an objective response rate of 16 % with a durable response rate of 4 %. Although IL-2 administration may induce toxicity owing to a capillary leak syndrome, treatment-related mortalities are less than 1 % [25].

Active immunization is another immunotherapeutic approach, which utilizes either whole cells, proteins, peptides or other immunizing vector that either increase immune recognition of tumor cells or enhances lymphocytic activation. Vaccines contained a single antigen specific to the target, or utilized a mixture of antigens such as Canvaxin, which contained over 20 tumor antigens. However, though up to 30 % of circulating anti-melanoma CD8+ T cells could be induced by immunization, tumors continued to progress and survival in stage IV patients was 5 % worse

while stage III patients was 9 % worse. The Canvaxin vaccine may have induced significant immunosuppression, which demonstrates the double-edged sword nature of complex vaccines [22, 23].

Another immunotherapeutic approach utilized anti-CTLA4 mAbs to blockade CTLA4 cell surface receptors in order to enhance T-cell activation, leading to a more robust antitumor immune response. CTLA4 is a negative regulator of T cell-mediated antitumor immune response and is the key element in immune tolerance. CTLA4 competes with its homolog, CD28, for binding to the B7 costimulatory molecule expressed on mature APCs. Binding of the CTLA4 receptor to B7 results in an inhibitory signal that downregulates T-cell activation, affecting downstream targets of CTLA4 including cytokine production by Th1 and Th2 cells and key cell cycle components (CDK4, CDK6, cyclin D3). However, not all patients benefit from the blockade treatment and response to the therapy has resulted in a grade III/IV autoimmune reaction such as colitis and dermatitis [23].

Pioneering work by several groups in recent years have overcome this low response rate associated with non-specific immunomodulation and active immunization approaches by using adoptive transfer T cell therapy (ACT) [22, 23]. They have demonstrated that antigen-specific T cells reactive to infectious pathogens and tumor antigens can be generated *in vitro* and adoptively transferred to patients providing a clinical benefit. Investigators at the National Cancer Institute (NCI) Surgery Branch led by Steven Rosenberg and our group at UCLA have utilized MART-1 TCR engineered α and β genes with high affinity for the melanoma tumor antigen MART-$1_{27\text{-}35}$ presented in the context of HLA A*0201. The transfer of TCR genes is necessary and sufficient to endow recipient T cells with the specificity of donor cells. TCR genetically modified T cells respond to target antigen recognition through the transgenic TCR both *in vitro* and *in vivo*, leading to effective immune responses to viral and tumor challenges in murine adoptive transfer models. T cells redirected by TCR gene transfer are fully functional after transfer into mice, and have been shown to expand dramatically (over three logs) after encounter with their cognate antigen *in vivo*, conferring new antigen specificity and functional activity to TILs. In addition, MART-$1_{27\text{-}35}$ pulsed dendritic cells are infused to ensure a more robust and sustained CTL activation [22, 23].

11.7 Molecular Targeted Therapy for the Treatment of Metastatic Melanoma Using $BRAF^{V660E}$-Specific Inhibitor Vemurafenib

Mitogen activated protein kinase (MAPK) family including the Extracellular signal-regulated kinase (ERK1/2) pathway control cancer cell proliferation, differentiation and survival. Receptor tyrosine kinases usually activate this signaling cascade. The GTPase RAS once actively GTP-bound, activates RAF (ARAF, BRAF or CRAF) by recruiting RAF to the plasma membrane activating mitogen activated protein kinase kinase 1/2 (MEK1/2), which consequently activates ERK1/2. BRAF is the

family member most easily activated by RAS and when mutated, signals as a monomer that is independent of upstream stimuli [26]. Various BRAF mutations occur in 60 % of patients with melanoma, in which 90 % of these mutations is the glutamic acid base substitution for valine at codon 600. The mutation leads to constitutive activation of the MARK pathway, which causes cancer cell proliferation and a 500-fold increase in activity compared to wild type protein [27].

Vemurafenib (PLX4032, RG7204), an oral serine-threonine kinase inhibitor, is BRAF ^{V600E} specific inhibitor. It preferentially inhibits the MARK pathway in melanoma and blocks phosphorylation of MEK and ERK leading to cell cycle arrest and apoptosis in cells exclusively harboring the BRAF ^{V600E} mutation [27].

MEK inhibitors also play an imperative role in inhibiting the MARK pathway in BRAF^{V600E} cells. U0126 and AZD6244 adequately block MEK and ERK 1/2 activation in melanoma cells. However, their benefits are often outweighed since they impair T-lymphocyte function [28]. Another MEK inhibitor is PD0325901 tested in phase I trials, produced significant decrease in ERK phosphorylation and disease stabilization [29]. Dabrafernib (GSK2118436) is another selective ATP competitive BRAF ^{V600E} inhibitor that is under development. Similar to Vemurafenib, the inhibitor has selectivity towards the mutant BRAF.

11.8 Molecular Mechanisms of Resistance to BRAF^{V660E}-Specific Inhibitor Vemurafenib

Progression free survival of melanoma patients treated with Vemurafenib is limited due to the developed resistance within an average of 6–8 months period [28, 29]. Various mechanisms are thought to play a role in drug-resistance. The mechanisms include the paradoxical hyperactivation of MAPK pathway, reactivation of mitogen-activated protein kinase kinase kinase 8 (COT), loss of phosphatase and tensin homolog (PTEN), PI3K/AKT/mTOR amplification, CRAF dimerization, suppression of BIM expression, increased cyclin D1, upregulation of N-RAS mutations, and high levels of platelet derived growth factor beta (PDGFRβ) and epidermal growth factor receptor (EGFR) [30]. Regulation of transcriptional events and phosphorylation through MAPK pathway causes cells to proliferate, avoid apoptosis, migrate and invade [28–30].

COT is MAPK pathway agonist with that causes resistance to the BRAF inhibitor through activation of MEK/ERK. In Vemurafenib-resistant melanomas, receptor tyrosine kinases (RTKs) are overexpressed. PDGFRβ and EGFR, contain extracellular ligand recognition and cytoplasmic tyrosine kinase domains. PDGFRβ displayed elevated activation-associated tyrosine phosphorylation and were positive for a melanoma marker, melanoma antigen recognized by T-cells 1 (MART-1). G0/G1 cell cycle arrest occurs when PDGFRβ is knocked down by shRNAs [30]. Activation of the receptor results in the activation of ERK pathway. Treatment of mutant cells with RTK inhibitor, gefitinib, inhibited growth of melanoma cells and decreased ERK phosphorylation. Resistant cells also express higher surface levels of insulin

growth factor receptor 1 (IGF-1R). Even though IGF-1R promotes the activation of PI3K, it has no effect on the MAPK pathway [28–30].

Deletion or functional loss of the tumor suppressor and negative regulator of PI3K/AKT pathway PTEN, which occurs in 5–20 % of melanomas, and upregulation or mutation in AKT allows the PI3K/AKT/mTOR signal transduction pathway amplification. PTEN is usually blocked by NRAS oncogenic mutations, mutations, silencing or AKT amplification. This further confers resistance to Vemurafenib and increased cell survival. Resistant cells have increased AKT3 signaling when exposed to PLX4032. AKT3 contains a point mutation E17K, which activates AKT pathway and influences mTOR activation. Receptor tyrosine kinases (RTKs) are upstream of P13K/AKT, such as IGF-IR and PDGFRβ, have phosphorylating activity, which might contribute to resistance [31].

NRAS activating mutations are detected in 15 % of melanomas, while KRAS and HRAS mutations are rare. The most common mutations are at codons 12 and 61, which lead to abnormal regulation of RAS and accumulation of RAF-GTP. Initiation of growth of melanoma cells is thought to be associated with RAS mutation. Some studies have shown Vemurafenib increases proliferation of growth factor dependent $NRAS^{Q61L}$ mutant in melanoma cells and increased mobility [28–30]. It was assumed that a resistant mechanism involving NRAS, was due to secondary mutation. However, even though NRAS is upregulated in Vemurafenib resistant cells, it is not due to the NRAS secondary mutation that would prevent the drug from binding to $BRAF^{V600E}$ [30].

MAPK signaling is hyperactivated by mutated NRAS that can activate CRAF, a RAF isomer, and BRAF heterodimerization and pathway switching, which can bypass Vemurafenib sensitivity. When BRAF mutants are impaired, CRAF activity is stimulated by BRAF through phosphorylation, and activated MEK signals to ERK [29]. It is also due to CRAF activation that Vemurafenib paradoxically induces the MEK/ERK pathway in BRAF WT cells even though inhibiting MEK/ERK phosphorylation in $BRAF^{V600E}$ tumors [32].

RAF/MEK/ERK MAPK pathway is also inhibited by Bim, a proapoptotic member of Bcl-2 family that binds with high affinity to antiapoptotic Bcl-2 proteins Bcl-2, Bcl-w, Bcl-XL and Mcl-1. BRAF inhibitor treatment also increases Mcl-1 levels, which is a pro-survival protein preventing apoptosis. ERK pathway inhibits apoptosis by phosphorylating Bad and Bim [33]. Bim inhibits the RAF/MEK/ERK pathway in melanoma, causing apoptosis. Phosphorylated Bim is proteasomally degraded and it no longer associates with Bax. Bad phosphorylation disrupts its interaction with antiapoptotic Bcl-2, which favors cancer cell survival [33]. Differences in Bim and Bad expression may allow some mutated cells to stop proliferating but not die from Vemurafenib. $BRAF^{V600E}$ inhibition triggers Bim(S) splicing by splicing factor SRp55, and when the mutation is expressed apoptosis and induction of Bim(S) decreases. Levels of Bim are determined by PTEN activation. Therefore, deregulation of PTEN reduces Bim binding and increases its suppression, which in turn will affect RAF/MEK/ERK inhibition. c-KIT is also known to contribute to Vemurafenib-resistance. c-KIT is a growth factor receptor in epidermal melanocytes. Its roles include migration and differentiation of melanocytes.

Abnormalities in c-KIT include gene mutations and amplification. Imatinib inhibits the tyrosine-kinase activity of c-KIT and induces tumor regression [34]. Reactivation of MAPK pathway due to Vemurafenib-resistance could also be caused by fibroblast growth factor receptor 3 (FGFR3). Enhanced receptor activity is linked to RAS and MAPK activation and its inhibition confers sensitivity. FGFR3/RAS signaling pathway is another mechanism of resistance that could deem favorable as a targeted therapy. Activation of FGFR3 controls RAS activation in $BRAF^{V600E}$ melanoma cell lines and reduces the sensitivity to BRAF inhibition, which results in cancer cell proliferation, growth and survival. Phospho-FGFR3 levels are increased in Vemurafenib-resistant cells specifically due to activation of downstream ERK. However, no phospho-AKT is detected in the melanoma cell lines conveying that the receptor does not take much part in the AKT pathway [35].

11.9 Epigenetic Modifications in Melanoma: Role of Chromatin Remodeling Drugs in Overcoming Acquired Resistance

Recent technological advances such as array-based high-throughput gene expression analysis in understanding the specific genes involved as well as the signal transduction pathways and the comparative gene expression patterns of primary and metastatic melanoma have provided unique opportunities to examine this deadly disease in greater depth. In Particular, these advances have presented opportunities to an improved understanding of the gene expression patterns involved with melanoma progression [36, 37]. Irreversible changes in the DNA sequence, including chromosomal deletions or amplification, activating or inactivating gene mutations, have been implicated in the development and progression of melanoma. However, growing attention is being paid towards understanding the implications of 'epigenetic' events in melanoma progression. Epigenetic events do not affect DNA sequence, but may lead to stable inherited changes in gene expression profile of tumor cells. Epigenetic events leading to abnormal gene expression in melanoma are usually due to histone modifications (acetylation and deacetylation), methylation of gene promoter regions, demethylation of CpG islands, and the role of microRNA. Thus, epigenetic mechanisms have emerged as playing a pivotal role in gene regulation of human melanoma, including the identification of several putative tumor suppressor genes (e.g., PTEN) and oncogenes (e.g., NRAS, BRAF). Further research will focus on the development of novel therapeutics that will likely target and alter such epigenetic changes [35–38].

As mentioned above, Adoptive cell therapy (ACT) of metastatic melanoma patients with T cell receptor (TCR)-engineered T lymphocytes results in dramatic clinical responses in a significant percentage of the patients; in recent clinical trials conducted at UCLA and Caltech by the Program in Engineered Immunity (PEI), nearly all patients demonstrated tumor regression, many quite dramatic,

Table 11.1 Focused array qPCR analysis of apoptosis genes modified by PLX4032 in M249 line (values represent fold change in the expression of the transcript)

Regulation of Apoptosis					
Positive		Negative		CARD Domain Proteins	
BCL2L11	4.68	CD40LG	2.81	CARD6	2.68
BIK	2.27	NOL3	2.33	CASP1	−11.33
BIM	4.68			CASP9	2.18
CASP1	−11.33			NOL3	2.33
TNFRSF10A	−2.11				
TNFSRF9	3.8			**BCL2 and BAG Proteins**	
TRADD	2.26			BCL2L11	4.68
TNF/TNFR Domain Proteins		**Death Domain Proteins**		**Caspases and Regulators**	
				Caspases	
TNFRSF10A	−2.11	TNFRSF10A	−2.11	CASP1	−11.33
TNFRSF9	3.8	TRADD	2.26	CASP9	2.18

but all recurred within months. Concurrent with these complex cell and gene therapy-based clinical investigations has been the clinical experience with the BRAF-targeted drugs (PLX4032, Vemurafenib) in which roughly 90 % of the patients demonstrated evidence of tumor regression but with a duration of response of about 6 months.

These clinical observations raise the obvious question whether there is a biological basis for combining immune- and BRAF-based therapies for this disease. Work from other groups has shown that BRAF inhibitors: (a) increase expression of melanocyte lineage antigens, (b) do not inhibit activated T lymphocytes, (c) also do not "sensitize" melanoma cells to T lymphocyte-delivered apoptotic death signals. Our group has approached this question using a melanoma cell line M249 which harbors $BRAF^{V600E}$, is MART-1 positive and transduced to express high levels of surface HLA A*0201: the M249 metastatic melanoma cell line is exquisitely sensitive to PLX4032 and MART-1 F5 TCR-engineered T lymphocytes (F5 CTL)-mediated apoptosis. Serial exposure of M249 over several months to PLX4032 or F5 CTL has yielded multiple completely resistant melanoma cell lines: M249(CtlR) and M249(PlxR). We have made the novel preliminary observation that M249(PlxR) are completely resistant to killing by F5 CTL, suggesting that both the drug and F5 CTLs kill the tumor cells share a common apoptotic pathway. This notion was further reinforced by the observation that the expression pattern of a wide array of apoptosis gene products are being modified upon treatment of the tumor cells with the drug PLX4032 (Table 11.1). As depicted in Table 11.1, SAHA negatively regulates the transcript levels of anti-apoptosis genes and simultaneously, positively regulates the expression of positive regulators of apoptosis (e.g., caspases, death domain proteins, TRAIL, TNF superfamily members, DNA damage molecules, apoptosis inducers). This suggests that SAHA mediates it sensitizing effect via

combinatorial cooperation among several groups of apoptotic genes (simultaneous reduction of anti-apoptotic and induction of proapoptotic genes). However, cell fate is ultimately determined by an imbalance in the ratio of pro- and anti-apoptotic proteins. We have reported the ability of SAHA to regulate (positively and negatively) the expression of apoptosis-associated genes [12]. Apparently, SAHA favors the generation of a proapoptotic milieu, which predestines the tumors to undergo apoptosis upon receiving apoptotic death signal delivered by F5 CTLs.

A regulatory role of the HDACis on the expression pattern of apoptotic genes rendering the cells more susceptible to apoptotic stimuli and in overcoming BRAF inhibitor resistance is reported [12]. Thus, it is logical to assume that treatment of dual resistant melanomas with physiologically relevant concentrations of HADCi, through modulation of apoptotic machinery, could potentially reverse the acquired resistant phenotype. To validate this approach, we initially used two HDAC inhibitors namely, LBH5089 and SAHA. Both of these agents are known to modify gene expression profile, in particular those involved in cell signal transduction pathways and apoptosis. Pretreatment of immune-resistant melanomas with subtoxic and clinically achievable concentrations of these HDACis rendered the cells sensitive to the cytotoxic effects of TCR engineered T cells (Fig. 11.1). Next, we tested the hypothesis that, through modulation of apoptotic machinery, SAHA can reverse the dual resistance of melanomas to both TCR engineered F5 CTLS as well as Vemurafenib. We observed that treatment of both M249(CtlR) and M249(PlxR) lines with the subtoxic and clinically relevant concentration of the FDA approved histone deacetylase inhibitor Vorinostat (Zolinza®, SAHA) restores sensitivity to F5 CTL killing (unpublished data). Preliminary focused microarray data suggests that this reversal of resistance is due to regulation of the expression levels of apoptotic gene products. Thus, chromatin remodeling drugs represent as new therapeutic modalities in the treatment of advanced and resistant melanomas which can be used as adjuvants to immunotherapy.

11.10 Concluding Remarks and Future Perspectives

Human metastatic melanoma, which is the deadliest form of skin cancer, is notoriously invasive, and best characterized by its resistance to almost all conventional treatments. Adoptive cell therapy (ACT) with T cell receptor (TCR)-engineered T lymphocytes (F5 MART TCR) represents a novel approach in melanoma immunotherapy. The F5 MART TCR, cloned at the NCI Surgery Branch by Dr. Steven Rosenberg, is of high affinity, both α and β chains preferentially assemble when retrovirally transduced into CD8 and CD4 T cells and confer HLA A*0201-restricted MART killing. F5 TCR-transduced human T cells (F5 CTL) are biologically very active in clinical trials conducted at NCI and by the UCLA/Caltech "Program in Engineered Immunity". As few as 10^9 cells were administered and nearly all melanoma patients show tumor regression, with many showing dramatic tumor regression. However, despite dense infiltration of tumor deposits by transgenic

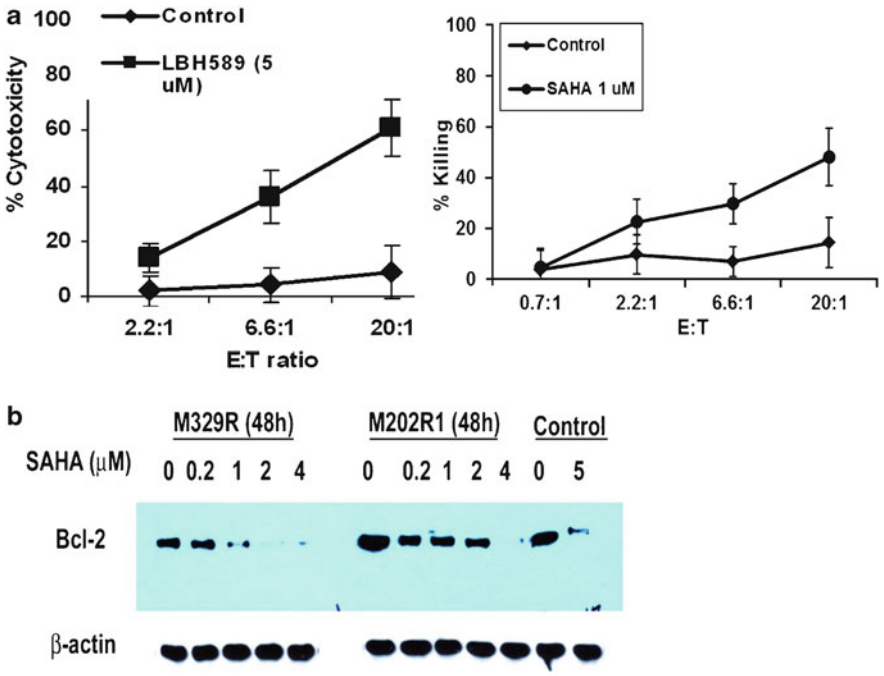

Fig. 11.1 (**a**) Immunosensitization of resistant melanoma line M329 (M329R) to F5 CTL-mediated killing by HDACi LBH 589 and SAHA. Tumor cells were either left untreated or treated with LBH589 or SAHA for 48 h. Then, they were used in 6 h standard 51Cr-release assay using F5 CTLs as effectors. (**b**) Time and concentration-dependent down regulation of anti-apoptotic Bcl-2 by SAHA in resistant cells as measured by western blot analysis

F5 CTLs, all patients relapsed within months. Concurrent with these complex cell and gene therapy-based clinical investigations has been the clinical experience with BRAF-targeted drugs. An activating mutation in the gene encoding protein kinase B-RAF^{V600E} that is found in about 60–70 % of melanomas and about 7 % of all human cancers, which plays a key role in melanoma growth and survival. The B-RAF^{V600E}–specific inhibitor PLX4032/Vemurafenib is the first FDA approved drug for molecular targeted therapy against advanced melanoma. Melanoma patients treated with PLX4032 show an unprecedented 84 % response rate, but acquired drug-resistance frequently develops within 6 months of the initial positive response, leading to a relapse and eventually the patients' demise.

We hypothesized that adoption of bypass/escape survival signaling pathways (e.g., AKT) and aberrant apoptotic machinery may confer resistance to death signals delivered by Vemurafenib as well as transgenic CTLs. To test this hypothesis, we established an *in vitro* model of resistant (R) lines from MART-1+/A*0210+ F5 CTL- and Vemurafenib-sensitive lines harboring BRAF^{V600E} under Vemurafenib-selective pressure. Interestingly, PLX-resistant tumors, while surviving high

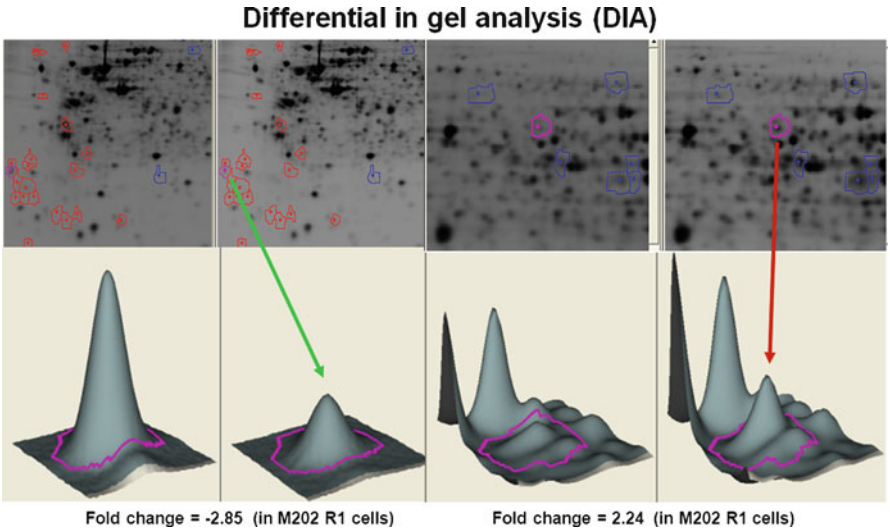

Fig. 11.2 Differential in gel analysis (*DIA*). Global quantification of protein expression between control and SAHA treated cells using 2D DIA in combination with mass spectrometry is a powerful tool for differential protein expression in PLX-resistant and identification of proteins regulated by SAHA. We will use this technology for proteomic analysis. M202R1 and M202R1 + SAHA were labeled with Cy3 (*red*) and Cy5 (*green*) & subjected to 2D gradient gel analysis (1–16 % SDS PAGE, pH: 3–11). Differential protein expressions (induced or reduced) as measured by fold change are shown

PLX4032 concentrations, develop cross-resistance to F5 CTL-killing, suggesting the use of a common apoptotic pathway by both modalities. Preliminary experiments also indicate that the acquired F5 CTL-resistance of PLX-resistant cells can be reversed with the histone deacetylase inhibitor (HDACi) Vorinostat (SAHA), a chromatin remodeling drug. However, the bypass and compensatory signaling pathways and the molecular determinants responsible for immune- and PLX-resistance are unidentified. Moreover, the exact underlying molecular mechanisms of SAHA-mediated immunosensitization are largely unknown. Future clinical investigations are needed to test whether the addition of an HDACi to $BRAF^{V600E}$-based targeted therapy will immunosensitize PLX-resistant metastatic melanomas patients to F5 CTL ACT. Further, using "differential in gel analysis" (DIA) (similar to Fig. 11.2) followed by mass spectrometry will identify the exact nature of proteins differentially expressed in PlxR lines and those modulated by SAHA. This will lead to the identification of molecular determinants and biomarkers of PLX- and/or immune-resistance as well as mechanism of sensitization by SAHA.

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Chapter 12
The Role of Epigenetics in Radiation Therapy and the DNA Damage Response

Steven P. Zielske

Abstract The impact of epigenetics in the field of radiation oncology and the DNA damage response is an emerging area of research. Epigenetic mechanisms may potentially play a role in inherent or acquired radioresistance of tumors. In this section, we will discuss what is known about epigenetics, specifically DNA methylation and miRNAs, with regards to the DNA damage response and the exploitation of epigenetics therapeutically. Very little is known about histone modifications and the DNA damage response. Current research in radiation oncology and epigenetics is now at the level of basic science, but is beginning to move to the level of pre-clinical and translational research. The speed of research is accelerating since there are currently epigenetic therapies approved for treatment of certain cancers outside of the radiation oncology clinic.

Keywords Radiosensitization • DNA methylation • DNA methyltransferase • miRNA • 5-azacytidine • Decitabine • Zebularine

12.1 DNA Damage Response

12.1.1 Micro RNAs

Micro RNAs (miRNA) are short RNAs that function post-transcriptionally to regulate protein expression. MiRNAs have been shown to regulate genes involved in tumorigenesis, apoptosis, angiogenesis, and other cancer cell activities [1]. MiRNAs can also regulate tumor suppressors. In the area of radiation, at this writing there

S.P. Zielske (✉)
Department of Radiation Oncology, Karmanos Cancer Institute, Wayne State University, 540 E. Canfield, 3343 Scott Hall, Detroit, MI 48201, USA
e-mail: szielske@med.wayne.edu

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are 319 PubMed entries for the search of “miRNA” AND “radiation”. Included in this set, 170 miRNAs have been predicted to target DNA-damage response genes. The number of miRNAs that have been experimentally determined to affect the DNA damage response is 174. The predicted and experimentally determined miRNA list are not identical. These statistics illustrate the early stage at which miRNA research is when it applies to radiation therapy.

A series of profiling studies suggests that exposure to ionizing radiation (IR) results in a response that includes altered expression of miRNAs. A screen of 1,090 miRNAs in human embryonic stem cells (hESCs) exposed to 1 Gy IR resulted in 54 miRNAs differentially expressed 16 h post-IR [2]. The number of up-regulated miRNAs outnumbered down-regulated miRNAs by 5:1. Gene ontology analysis of the predicted target genes for down-regulated miRNAs was performed and overrepresented categories included cell cycle, cell death, transcription, and cell differentiation. Changes in miRNA expression following radiation may represent a broad and specific response that influences DNA repair or cell survival. In one study of miRNAs differentially expressed 30 min and 2 h after radiation, the data suggests down-regulation of miRNAs controlling expression of genes involved in DNA repair [3]. This would be expected to increase expression of DNA repair genes and potentially enhance DNA repair. At 2 h, however, there was an up-regulation of miRNAs controlling expression of apoptosis-related genes – expected to result in decreased expression of apoptosis genes. This is hypothesized to increase cell survival.

The let-7 family of miRNAs are differentially expressed following radiation [4–8]. However, agreement on the direction of change is lacking. This may be partly explained by different cell types, radiation doses, and post-IR analysis times. Let-7f-2 is down-regulated 8 h following 50 cGy treatment in a lymphoblast cell line [5]. Let-7g is up-regulated 6 h after 2 Gy in normal endothelial cells [4]. Let7a-i were up-regulated in Jurkat cells at 4–24 h following 2 Gy, but in TK6 cells (B lymphoblast cells), most let-7 members were down-regulated [7]. Let-7e was up-regulated 3–8 h following 10–400 cGy in normal human skin fibroblasts, while other let-7 family members showed only modest changes [6]. Fractionated radiation treatment of prostate cancer cells also results in increased let-7 miRNA [8]. Let-7 family miRNAs negatively regulates the RAS oncogene, suggesting a mechanism for an influence on radiosensitivity. Over-expression of let-7g in irradiation of endothelial cells results in decreased clonogenic survival, while inhibition of let-7g increases clonogenic survival [4]. This study provides an important link between radiation-induced changes in let-7g expression and the radiation response. If radiation causes increased let-7 miRNA expression and this decreases clonogenic survival, it would not seem that this effect is a protective mechanism for the cell. One possibility is that this response pushes cells with poorly repaired DNA or that have become genomically unstable toward apoptosis.

The effect of radiation on expression of miR-20 and miR-21 are equally variable. Low radiation doses (10–400 cGy) have been shown to up-regulate or down-regulate

expression [5, 6]. Up-regulation was observed at 2 Gy [4]. Inhibition of miR-20a had little effect on clonogenic survival following IR. When a biological consequence of the differential miRNA expression can be found, more weight can be assigned to these observations.

Differential expression of miRNAs can be detected in peripheral blood cells of patients following radiotherapy [9]. Three hours following a total body irradiation of 1.25 Gy, let-7f, let-7g, miR-20a, and miR-21 were among 45 up-regulated miRNAs found. Thirty-eight miRNAs were found to be differentially expressed in all seven patients. Detection of miRNAs in peripheral blood cells could be used to test for radiation exposure.

Studies targeting specific miRNAs and the effect on radiation resistance have measured biological effects. MiR-148b is up-regulated by IR and overexpression radiosensitized lymphoblastoid Raji cells by enhancing apoptosis [10]. MiR-34b overexpression radiosensitized lung cancer cell lines but no difference in apoptosis was observed [11]. Induction of miR-99 by IR prevented an increase in the chromatin remodeling factor SNF2H [12]. Recruitment of BRCA1 to DNA damage sites was also reduced, leading to reduced repair efficiency.

PTEN is downregulated in late stage nasopharyngeal carcinoma and expression is regulated by miR-205. Following radiotherapy, miR-205 is up-regulated and ectopic overexpression reduced radiation-induced apoptosis [13]. The conclusion is that miR-205 expression contributes to radioresistance through PTEN, and its induction by radiotherapy may confer some degree of radioresistance on cells. The correlation between miR-205 and radioresistance suggests miR-205 could be used as a biomarker for determining the response to radiotherapy.

MiR-18a expression down regulates ATM expression [14]. ATM is an early sensor of DNA damage following IR and initiates signaling to result in DNA repair. In primary breast cancer tissue, miR-18a was found to be up-regulated and ectopic expression of miR-18a in breast cancer cells radiosensitized them through reduced DNA repair capacity. This potentially important finding demonstrates that miRNAs that regulate DNA repair could be new therapeutic targets.

These studies on miRNAs and radiation therapy hold promise in explaining contributors to radioresistance and offer potential novel targets for therapeutics. However, an interesting study that shows more work needs to be done investigates the effect of knockdown of the machinery required for miRNA processing and activity on radiation sensitivity [15]. Knockdown of Dicer, Drosha, and Ago2 did not result in radiosensitization of lung cancer cells. These proteins are essential for knockdown of mRNAs by miRNA or siRNA. One caveat to all RNAi experiments is whether the degree of knockdown is sufficient to observe a biological effect. Remaining low levels of the target protein may be enough to maintain much of the activity one is attempting to eliminate. This study used apoptosis as a measure of radiation sensitivity. Clonogenic survival is a more robust assay for determining the impact of radiation. This may account for the differences between the studies described.

12.1.2 DNA Methylation

There is little information on DNA methylation response following exposure to IR or the impact of DNA methylation on the DNA damage response. It has been shown that IR results in global DNA hypomethylation in some normal tissues, but not others, as well as in cell lines [16–18]. Others have failed to find significant changes in DNA methylation in normal cells exposed to radiation after a 7 day recovery [19]. Decreased global DNA methylation was observed following fractionated radiation and this was accompanied by a loss of histone H4-Lys20 trimethylation [20]. These data were later extended to show locus-specific changes in DNA methylation following regrowth of MCF7 breast cancer cells following IR [21]. These changes were found 14 days following irradiation and included differential DNA methylation of FOXC1 and TRAPPC9. Our group has found global loci-specific changes in DNA methylation following IR (unpublished data). This response initiated as soon as 1 h after IR and extended over 3 days, and varied according to radiation dose. The preponderance of evidence thus far suggests either a DNA methylation response to IR and/or an involvement in DNA methylation in radioresistance. However, the functional significance of global changes in DNA methylation is yet to be deciphered.

Hyper- or hypomethylation of specific genes may partly account for the radioresistant phenotype. DNA methylation profiles have been compared in radiosensitive and radioresistant cells and suggest involvement in maintenance or induction of radioresistance [18, 22]. In a comparison of a radioresistant and radiosensitive lung cancer cell line, 1,091 differentially methylated genes were discovered [22]. Among these, SERPINB5 and S100A6 were found to be hypermethylated in the radioresistant cell line while CAT and BNC1 were hypomethylated in the radioresistant cell line. SERPINB5 and S100A6 were more highly expressed in the radiosensitive line and upon RNAi, the cells became more radioresistant. Conversely, RNAi of CAT and BNC1 in the radioresistant cell line sensitized them to IR. This study is limited by the fact that two different cell lines were compared instead of isogenic cell line pairs. It is likely that many differences in DNA methylation between the two cell lines exist without an impact on radiation resistance. In fact, in the two cell lines compared, one was p53 wild type and the other was p53 negative. Nevertheless, the genes investigated had some impact on radioresistance under their experimental context. Additional studies using other models may be more revealing.

12.2 Radiosensitization by DNA Demethylation

A number of compounds demethylate DNA by inhibiting DNA methyltransferases, in particular DNA methyltransferase 1 (DNMT1). DNMT1 is the predominant DNMT that methylates DNA following replication. DNMT inhibitors fall

into two classes: nucleoside analogs (5-azacytidine, 5-aza-2′-deoxycytidine, 5,6-dihydro-5-azacytidine, zebularine) and non-nucleoside analogs (hydralazine, RG108, procainamide, procaine, SGI-1027). There is active research taking place to develop new inhibitors of both the nucleoside analog and non-nucleoside analog classes due to some of the caveats described below [23, 24].

DNMT inhibition by nucleoside analogs occurs through incorporation into the DNA whereby the DNMT is trapped by the modified pyrimidine and targeted for degradation by the proteasome [25]. A decrease in DNMT1 leads to lower levels of DNA methylation of newly synthesized DNA during cell proliferation. Thus, DNA demethylation results not from active removal of methyl groups from DNA, but more accurately, the dilution of existing methylated DNA by newly synthesized unmethylated DNA. DNA replication is a necessary requirement for the DNA to become demethylated. Toxicity in this class is greatest with 5-azacytidine (5AC). This may partially be due to the fact that 5AC can be incorporated into both DNA and RNA and thus has some effect in non-dividing cells. Decitabine (5-aza-2′-deoxycytidine) and zebularine have lower toxicity. Zebularine, 5AC, and decitabine have been approved for treatment of myelodysplatic syndromes.

The mechanisms of non-nucleoside analog inhibitors of DNMT are more diverse. Procaine prevents DNMT binding to CpG DNA sequences by binding those sequences itself [26]. Hydralazine and procainamide are thought to have similar mechanisms but may also interact with amino acid residues in DNMT and inhibit catalytic activity. RG108 is a rationally designed inhibitor of DNMT and unlike other inhibitors does not require DNMT to bind DNA to exert its activity. RG108 has low cytotoxicity and is considered a promising candidate to bring forward.

Many tumors are resistant to radiation and ways to enhance the effectiveness of treatment by reducing tumor radioresistance is an avid area of study. The effectiveness of radiotherapy is a function of the radioresistance of the tumor versus the radiosensitivity of normal tissue which limits radiation dose. Thus, drugs which decrease the inherent radioresistance of tumors potentially improve the therapeutic ratio of radiotherapy.

Zebularine was the first DNMT inhibitor to be shown to radiosensitize cancer cells [27]. Pancreatic, glioblastoma, and prostate cancer cell lines were exposed to zebularine for 24 or 48 h before irradiation. Clonogenic assays were used to assess radioresistance and it was found that incubation with zebularine for 24 h resulted in an average radiation enhancement factor of 1.2, which is a moderate increase in radiation sensitivity. However, after a 48 h incubation with zebularine a high level of radiosensitization was observed, with an average radiation enhancement factor of 1.6. In glioblastoma xenografts, treatment with 350 mg/kg zebularine every 8 h for 3 days led to a significant tumor growth delay that was equivalent to a single 4 Gy dose of radiation. When zebularine and radiation were combined, the growth delay was significantly longer. No obvious toxicity was observed in mice treated with zebularine.

Although the first study combining a DNMT inhibitor with radiation was done with zebularine, subsequent studies by other groups have predominantly investigated 5AC and decitabine. Hofstetter et al. [28] demonstrate strong radiosensitization of

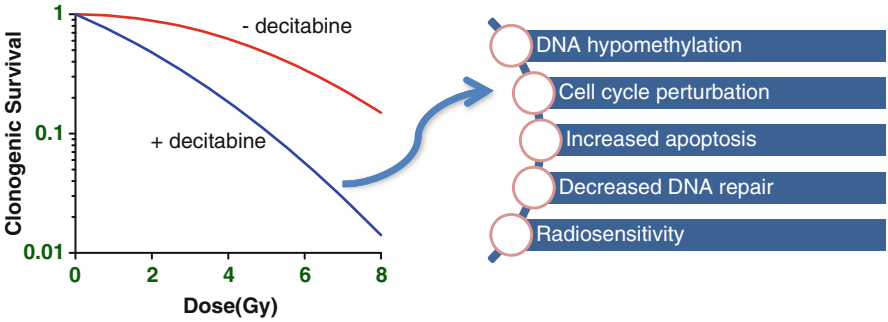

Fig. 12.1 Decitabine treatment in combination with radiation reduces clonogenic survival and induces radiosensitivity by causing DNA hypomethylation, cell cycle perturbation, increased apoptosis, and decreased DNA repair

colorectal carcinoma by 5AC *in vitro*. Decitabine has been shown to radiosensitize breast, head and neck, and gastric cancer cell lines [29–31]. In gastric cancer, 2 of 4 cell lines showed modest radiosensitization and in the breast cancer study, MDA-MB-231 cells were strongly sensitized. A study of medulloblastoma cell lines, however, showed no increased radiosensitivity following decitabine treatment [32]. One concern about negative results is that 5AC and decitabine have a very short half-life in solution. The half-life of 5AC at 37 °C may be as short at 7 h [33]. This means that *in vitro* studies may require addition of fresh drug multiple times over a several day experiment. Storage and handling of the drugs are also important in order to maintain activity over many months. Head and neck cancer cell lines have been shown to be radiosensitized by decitabine and the effect was enhanced further by combined treatment with a histone deacetylase inhibitor [31]. The above *in vitro* studies have not yet been followed up with careful animal studies to determine the effect *in vivo*.

The mechanism of radiosensitization is still to be determined (Fig. 12.1). Since DNMT inhibitors reduce global DNA methylation, it has been hypothesized that they may alter expression of genes related to cell cycle or the DNA repair capacity of a cell. Some evidence supports a role for a change in cell cycle. An increase in G_2/M cells is observed in many cell lines following decitabine or zebularine treatment [29, 31], but this did not correlate with the radiosensitization effect seen by clonogenic assay [27, 30]. In colorectal cancer cells, 5AC alone did not cause a G_2 arrest but did potentiate the G_2-arrest seen after radiation treatment [28]. In contrast, zebularine has been shown to inhibit the G_2 arrest induced by radiation [34]. These disparate reports ultimately lend little support to the hypothesis that DNMT inhibitors radiosensitize cells due to an influence on cell cycle. Additional studies may shed more light on this question.

Cells treated with decitabine may become more susceptible to apoptosis triggered by radiation. When gastric cancer cells were treated with decitabine alone, no increase in apoptosis was observed, but when decitabine was combined with

radiation, the percentage of cells undergoing apoptosis increased [30]. Furthermore, cell lines which exhibited radiosensitization also showed increased apoptosis. DNMT inhibitor effects on apoptosis sensitivity are commonly cited in studies of DNMT inhibitors used alone for treatment of cancer [35].

The other potential mechanism of radiosensitization that has been investigated is the DNA repair activity in the cell (Fig. 12.1). The formation and resolution of nuclear γH2AX foci is an indicator of DNA damage signaling and the repair process. If DNA is repaired slowly, the time required for γH2AX resolution is delayed. Under normal conditions, γH2AX foci are largely resolved at 24 h post-irradiation. In cancer cells treated with radiation combined with decitabine or zebularine, however, γH2AX foci were still present after 24 h [27, 29, 34]. That delayed kinetics were observed by multiple groups across disparate cell lines and inhibitors strengthens the hypothesis that the ability of cells to repair DNA damage is impaired by DNMT inhibitors or the associated changes in gene expression that accompany DNA demethylation.

DNMT1 may not be the primary target through which DNMT inhibitors affect radiosensitization. DNMT1 deficient cells were no more sensitive to radiation than parental cells carrying wild type DNMT1 [28]. However, cells deficient in DNMT3b were more radiosensitive than their parental counterparts. The caveat to this study is that global DNA methylation differences between the cell lines were not measured. Nor was there a measurement of specific gene methylation differences. It is important to resolve this issue as studies go forward since the DNMT inhibitors have different activity towards DNMT1, DNMT3a, and DNMT3b [34].

Although DNA methylation is globally reduced by treatment with zebularine and other DNMT inhibitors, the effect on a gene-by-gene basis is variable. The 14-3-3σ and RASSF1A gene promoters are demethylated in breast, prostate, pancreatic and glioblastoma cells following treatment with zebularine or decitabine [27, 29]. Both of these genes are tumor suppressors that play a role in cell cycle and/or the DNA damage response through the various signaling pathways they interact with. Promoter demethylation was associated with an increase in gene expression [27]. In colon cancer cells, promoter regions for p16, hMLH1 and hTERT were demethylated by 5AC treatment and persisted for 7 days following removal of drug [28]. In studies focusing on gene expression, p53, caspase 6, DAPK1, DAPK2, and DAPK3 were shown to be increased following decitabine treatment. Only DAPK1 was increased by radiation alone but the highest expression of all five genes was observed under the combined treatment of decitabine and radiation [30]. The DAPK proteins are involved in cell survival, apoptosis, and autophagy. These data lend further support to increased susceptibility to apoptosis as a potential mechanism of DNMT inhibitor radiosensitization.

DNMT inhibitors that lead to global DNA demethylation are being studied as a class of drugs with radiosensitizing activity in a variety of cancers. Some of these compounds are already approved for treatment of some malignancies in their own right and new inhibitors are being developed with a more favorable stability and toxicity profile. The mechanism of radiosensitization remains elusive but may center around DNA repair and apoptosis.

12.3 Conclusions

DNA methylation and miRNAs clearly impact the radiation response and influence the radiation resistance of cancer cell *in vitro*. The challenge is determining how epigenetics may be manipulated to enhance the effectiveness of radiation therapy. Specific targeting of the effect may still be an issue with interventions utilizing epigenetic therapy, however, the opening of a new angle of attack against cancer offers opportunity.

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Chapter 13
Plant Polyphenols as Epigenetic Modulators of Glutathione S-Transferase P1 Activity

Vijay S. Thakur and Sanjay Gupta

Abstract Glutathione S-transferase P1 (GSTP1) is an enzyme that catalyzes the conjugation of glutathione and thioester bond formation to a variety of electrophilic substances, engaged as a housekeeper in the detoxification of xenobiotics. GSTP1 is abundantly expressed in some mammalian tissues and has been shown to act as a modulator of signal transduction pathways controlling proliferation and cell death. Loss of GSTP1 in normal tissues incurs increased oxidative damage to cells, making them susceptible to neoplastic transformation. In contrast, its overexpression in tumor cells leads to the development of resistance to anticancer agents. Use of dietary polyphenols and synthetic compounds to induce GSTP1 is a potential strategy for cancer prevention in humans. This chapter presents the current body of knowledge regarding GSTP1 and its roles in carcinogenesis, and highlights the need for further investigation of its potential in chemoprevention.

Keywords Glutathione S transferases • Plant polyphenols • Green tea • Cancer • Signal transduction • Xenobiotics

V.S. Thakur
Department of Urology, Case Western Reserve University,
10900 Euclid Avenue, Cleveland, OH 44106, USA

S. Gupta, Ph.D. (✉)
Department of Urology and Nutrition, Case Western Reserve University,
10900 Euclid Avenue, Cleveland, OH 44106, USA

University Hospitals Case Medical Center, Cleveland, OH, USA

Division of General Medical Sciences, Case Comprehensive Cancer Center,
Cleveland, OH 44106, USA
e-mail: sanjay.gupta@case.edu

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Abbreviations

GSTs	Glutathione S-transferases
GSH	Reduced glutathione
PGA2	Prostaglandin A2
GSTα or GSTA	Glutathione S transferase alpha or A
GSTμ or GSTM	Glutathione S transferase mu or M
GSTπ or GSTP	Glutathione S-transferase pi or P
GSTσ or GSTS	Glutathione S-transferase sigma or S
GSTθ or GSTT	Glutathione S-transferase theta or T
GSTω (omega) or GSTO	Glutathione S-transferase omega or O
GSTζ or GSTZ	Glutathione S-transferase zeta
PPAR γ	Peroxisome proliferators-activated receptors
GPE1	GSTP enhancer-1
C/EBPα	CCAAT enhancer-binding protein alpha
MOZ	Monocyte leukemia zinc finger protein
Sp1	Specificity Protein 1
NF-κB	Nuclear Factor-κappaB transcription factors
AP1	Activator Protein 1
H_2O_2	Hydrogen peroxide
TPA	12-*O*-tradecanoylphorbol-13-acetate
TBQH	Tert-butylhydro-quinone
TNF-α	Tumor necrosis factor alpha
miRNA	microRNA
DNMTs	DNA (cytosine-5-)-methyltransferases
EZH2	Enhancer of zeste homolog 2 (Drosophila)
HDAC-1	Histone deacetylase 1
JNK	c-Jun NH2-terminal kinase
TRAF2	TNF receptor-associated factor 2
ASK1	Apoptosis signal-regulating kinase 1
MBD	Methyl-CpG-binding domain
8-OHdG	8-oxo-2′-deoxogunosine
ROS	Reactive oxygen species
MAPK Kinase	MAP kinas*e* kinase
MRP1	Multidrug resistance proteins 1
MRP2	Multidrug resistance proteins 2
HDACs	Histone deacetylases
EC	Epicatechin
EGC	Epigallocatechin
ECG	Epicatechin-3-gallate
EGCG	Epigallocatechin-3-gallate
NDEA	N-nitrosodiethylamine
PH	partial hepatectomy
CCL_4	Carbon tetrachloride

GTP	Green tea polyphenols
SFN	Sulforaphane
PEITC	Phenethyl isothiocyanate
BITC	Benzyl isothiocyanate
Nrf2	Nuclear factor erythroid 2-related factor 2
PBITC	4-phenylbutyl isothiocyanate
PHITC	6-phenylhexyl isothiocyanate
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
D3T	3H-1,2-dithiole-3-thione
oltipraz	5-[2-pyrazinyl]-4-methyl-1,2-dithiol-3-thione
ADT	5-[4-methoxyphenyl]-1,2-dithiole-3-thione anethole dithiolethione

13.1 Introduction

Glutathione S-transferases (GSTs) belong to a multi gene enzymes family of phase II detoxifying enzymes of the xenobiotic metabolism [1]. The members of this family of dimeric enzymes are identified on the basis of their substrate specificity and amino acid sequences [2]. Glutathione S-transferases catalyze reactions in which reduced glutathione is conjugated to toxic oxidizing compounds. These compounds are produced either secondary to normal cellular activity of the cell or due to exposure of cells to xenobiotics and environmental pollutants including carcinogens, pesticides, drugs and to endogenous molecules. This conversion significantly detoxifies them by reducing their ability to react to cellular macromolecules [3]. These enzymes are ubiquitously present in every cell and in every living species examined, including both eukaryotes and in prokaryotes [4]. Although, most of these enzymes are composed of cytosolic proteins, a small family of microsomal and mitochondrial (kappa) GSTs also exist. GSTs are regarded as cell housekeepers due to their ability to detoxify both endogenous as well as exogenous cell substances [5]. In some mammalian and rodents organs, cytosolic GSTs can constitute as high as 4–10 % of cytosolic proteins [6]. Soluble cytosolic GSTs exist as dimeric proteins, possessing a molecular weight of approximately 25 kD of each subunit. Each subunit has an active site which is composed of two distinct functional groups. This includes a hydrophilic catalytically independent active G-site which binds to glutathione, a physiological substrate of GSTs and an adjacent H-site which provides a hydrophobic environment for binding of electrophilic substrates with diverse structures [7]. Whereas the G-site, which is in the amino terminal domain, is highly conserved among GSTs due to its high specificity for GSH, the H-site, which is in the carboxy-terminal domain, can be very divergent among GSTs, exhibiting broad and variable specificity to substrate binding. GSTs catalyze the conjugation of reduced glutathione (GSH) via a sulfhydryl group to electrophilic centers on substrates with variable binding specificity [8]. This activity detoxifies several reactive endogenously produced molecules such as α, β-unsaturated keto prostaglandins (i.e. PGA2), and endogenous fatty acid oxidation

products including 4-hydroxy-2-nonenal, peroxidized lipids and xenobiotics [9, 10]. GSTs were designated as ligandins, due to their ability to function as transport proteins to toxins [11, 12].

13.1.1 Glutathione S-Transferase Family Members

The mammalian GST super-family consists of seven classes of cytosolic GSTs, based on their amino acid sequence similarity, substrate specificity and immunological cross-reactivity. The family consists of GSTα (alpha), GSTμ (mu), GSTπ (pi), GSTσ (sigma), GSTθ (theta), GSTω (omega) and GSTζ (zeta) or GSTA, GSTM, GSTP, GSTS, GSTT, GSTO and GSTZ [13–15]. As functional GST enzymes are dimeric and the GSTα (GSTA) and GSTμ (GSTM) can form heterodimers in addition to homodimers, the number of isoenzymes in each class is large. Isoenzymes are named after their class and composition of subunits [16, 17]. Each subunit is designated as a numeral, for example the enzyme consisting of subunit 1 and 2 of the μ class is denoted as GSTM1-2 [17]. The expression of different GST classes during development stages and among different tissues differs significantly. While the levels of α-class GSTs are similar in adult and fetal tissue, the levels of GSTπ in the liver decrease during development and are very low in adult tissues. Whereas, α-class GSTs are expressed predominantly in liver, kidney and testis, π-class is found mainly in brain, lung and heart [18–20].

13.1.2 Glutathione S-Transferase P1

One of the highly conserved classes of cytoplasmic GST is glutathione S-transferase Pi (GSTP1), the predominant isoenzyme [21]. It is mapped to chromosome 11q13 and its 4 allelic variants have been described [22]. The genes of this class are about 3 kb long and contain seven exons [23]. GSTP1 protects cells from cytotoxic and carcinogenic agents. It is expressed at variable levels in different cell types in normal tissues and its altered activity and expression has been found to play an important role in determining susceptibility to different type of cancers, inflammatory disorders, asthma and neurodegenerative disorders [24–27]. A vast majority of human tumor cell lines over-expresses GSTP1, including cells selected *in vitro* for resistance to agents used for chemotherapy. In 58 of the 60 human tumor cell lines used in the Drug Screen Program of the National Cancer Institute, GSTP1 was found to be the predominant isoenzyme (as high as 2.7 % of the total cytosolic protein). A significant quantitative correlation among enzyme activity, protein and mRNA were shown particularly in those cell lines selected for resistance to alkylating agents [28]. Such comparable correlation was much less apparent for overexpression of GSTA and GSTM [28].

13.1.3 GSTP1 Polymorphisms

Numerous studies, in the past few years have identified genetic polymorphisms of GSTP1 and their association with different cancers including prostate cancer [29–34]. Two single nucleotide GSTP1 polymorphisms are frequently described: the A → G transition in the GSTP1 gene which results from a change in the amino acid from isoleucine to valine at codon 105; and C → G substitution resulting a change in the amino acid alanine to valine at codon 114, thus generating four GSTP1 alleles: wild type GSTP1*A (ILe105/Ala114), GSTP1*B (Val105/Ala114), GSTP1*C (Val105/Val114) and GSTP1*D (Ile105/Val114), respectively [33]. As isoleucine amino acid is present in the substrate binding site of GSTP1, its change to valine in individuals with a GSTP1*B allele is associated with significantly decreased GSTP1 enzyme activity and detoxification ability and increased susceptibility to various diseases, including cancer [35]. Additionally, homozygocity for GSTP1*B is linked with diminished ability to detoxify platinum-based chemotherapeutic agents [36].

13.1.4 GSTP1 Regulation

Adequate GSTP1 expression is important to the cell and is regulated by transcriptional activation and repression and by post-translational modifications [37]. Like most enzymes, GSTP1 activity is regulated through protein expression and temperature [38]. GSTP1 expression and activity is also affected by the redox state of the cell, as oxidative stress decreases GSTP1 activity by formation of disulfide bonds between cystine 47 and 101 [39]. Treatment of cells with H_2O_2 leads to GSTP1 upregulation whereas treatment with N-acetyl cysteine downregulates GSTP1 expression [40]. GSTP1 is induced by a group of chemoprotective agents such as oltipraz, sulforaphane, diindole methane, and 1,2-dithiole-3-thione. A number of endogenous and exogenous compounds including xenobiotics, chemotherapeutic agents, anti-estrogens and various toxicants or prooxidants upregulate GSTP1 expression [41]. In addition, various growth factors including insulin, insulin-like growth factor and peroxisome proliferators-activated receptors (PPAR) γ agonists increase the level of GSTP1 expression [42].

Transcription of the GSTP1 gene is regulated by a number of transcription modulators including transcription factors (Fig. 13.1). The promoter region of GSTP has revealed 4 putative transcription regulatory motifs. These include a ‘TATA’ box 29 bp upstream from the major transcription start point (nt position -29), 2 Sp1 recognition sequences (-46 to -41 and -56 to -51), an AP-1 recognition sequence (-69 to -63), a NF-κB p50/65 and p65/p65 dimers binding site (-323 to -314) and a negatively acting regulatory element (-105 to -86) [43–45]. The first 200 nt 5′ to the start point of transcription contain a G+C rich region (79 %) [43]. An enhancer element GPE1 (GSTP enhancer-1) is reported to regulate the expression of GSTP gene by interacting with specific transcription factors in normal liver and during rat

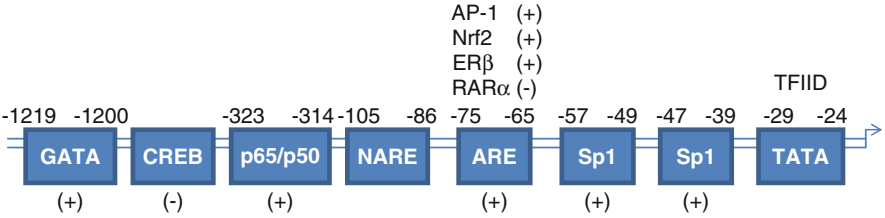

Fig. 13.1 5′-regulatory region of the human GSTP gene and binding sites for different transcription modulators. (+) demonstrates positive regulation, (−) demonstrates negative regulation

hepatocarcinogenesis [46]. Reports suggest that CCAAT enhancer-binding protein alpha (C/EBP α) is required for GSTP gene suppression in normal liver [47], whereas Nrf2/MafK heterodimer in association with monocyte leukemia zinc finger protein (MOZ) is required for GSTP gene activation during hepatocarcinogenesis [48]. Butyrate dependent suppression of GSTP transcription may depend on the GATA sequence located at -1208 relative to the transcriptional start site of human GSTP promoter [49].

Studies have demonstrated that transcription factors Activator Protein 1 (AP1), Specificity Protein 1 (Sp1) and NF-κB mediate GSTP1 regulation by redox processes [44]. It has been shown that transcriptional activity of GSTP1 depends on AP1 binding elements within the promoter region of GSTP1 gene [45, 50]. Exposure of K562 cells to various inducers of oxidative stress such as H_2O_2, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as well as chemotherapeutic agents viz. TBQH and doxorubicin leads to increased binding of AP1 to GSTP1 promoter on AP1 binding element [51]. Expression of GSTP1 is also shown to be regulated by inducible AP1 binding in leukemic cells [50]. Retinoic acid and insulin has been reported to suppress GSTP1 expression by binding to consensus AP1 binding site via binding to human beta-type retinoic acid receptor in human bladder carcinoma EJ cells, and in human breast carcinoma MCF-7 cells [52]. Studies have shown that constitutive expression of GSTP1 during Caco-2 cell differentiation is controlled by CDX2 binding to the putative consensus CDX-binding element within human GSTP1 promoter via formation of a complex with Sp1 [53]. TNFα treatment as well as co-transfection of NF-κB signaling pathway intermediates induced activation of the GSTP1 gene promoter in K562 leukemia cells. It has been shown that NF-κB p50/65 and p65/p65 dimers bind to a sequence located at -323/-314 in the GSTP1 promoter and are involved in the regulation of the gene by TNFα [44]. Chemopreventive agents including β-lapachone, emodin, sanguinarine and capsaicin, significantly inhibited GSTP1 expression as well as TNFα-and TPA-induced binding of AP1 and NF-κB on GSTP1 promoter in K562 and U937 leukemia cells [51]. In another study, of both primary tumors and cell lines of human malignant gliomas, p53, a major tumor suppressor and transcription factor was reported to bind to a putative p53-binding motif located in intron four of the *GSTP1* gene spanning nucleotides +983 and +1002 in the *GSTP1* gene, regulating GSTP1 expression and protecting the genome from alkylating and free radical generating compounds [54].

In tumors, transcriptional activation of the *GSTP1* gene by p53 has been shown to increase survival and drug resistance, whereas in normal cells it leads to protection against genotoxins [54].

Expression of the GSTP1 gene is also regulated by epigenetic modulations such as hypermethylation of its promoter, modifications in histone proteins and post-translational alterations in miRNA profile. GSTP1 is affected by *de novo* methylation during prostate carcinogenesis [55]. Most primary prostate cancers do not express GSTP1 which is in contrast with most other human cancers, which overexpress GSTP1 [56, 57]. GSTP1 over-expression has been observed in sarcomas, germ cell tumors (embryonal carcinoma of testis) and in cancers of the breast, colon, stomach, lung, bladder, cervix, ovary, pancreas, brain, and the head and neck [7]. Hypermethylation of GSTP1 promoter has been reported in >90 % of the primary prostate cancers in the majority of published studies [58, 59]. Hypermethylation was not associated with elevated levels of DNA methyltransferases, removal of AP1 transcripition factor binding sites in the CpG island or CpG island boundary elements or prior gene silencing in a study using human prostate cancer LNCaP and DU145 cells. The results of the study support a model that illustrates a combination of prior gene silencing and random seeds of methylation to trigger hypermethylation of GSTP1 gene in prostate cancer [60].

Increased expression of EZH2, a histone methyltransferase and histone deacetylases (HDACs) in prostate cancer may also increase *de novo* methylation of GSTP1 promoter, since both EZH2 and class I HDACs have been documented to cause increase expression and activity of DNA methyltransferases [61–63]. A recent study has shown that upregulation of a p53 downstream target, maspin, upregulates GSTP1 expression by downregulation of HDAC1 in prostate cancer [64]. In addition, GSTP1 expression is also regulated by miRNAs. Increased expression of miRNA133a and miRNA133b has been reported to cause decreased expression of GSTP1 in lung, bladder, prostate and head and neck cancers [65–68].

13.1.5 Regulatory Functions of GSTP1

In addition to its enzymatic functions, GSTP1 also independently functions as a modulator of signaling pathways (Fig. 13.2). It has been shown that GSTP1 interacts with c-Jun NH2-terminal kinase (JNK) and suppresses its activity [69]. Inhibition of JNK activity by GSTP1 was found primarily in normal growing non-stressed cells [70]. GSTP1 also has a role in regulating the constitutive expression of specific phase II detoxification enzymes and antioxidant proteins that are downstream molecular targets of the JNK signaling pathway and thus may serve as a sensor of intracellular changes in redox potential elicited by stress of different forms [71]. GSTP1 directly associates with TRAF2, and subsequently inhibits the TRAF2-elicited ASK1–JNK signaling cascade and may regulate the TNFα-activated TRAF2–ASK1 axis and subsequent cell apoptosis by interacting with TRAF2 [72]. GSTP1 deletion mutant (Delta194-209) and a site-directed mutant (R201A) in the

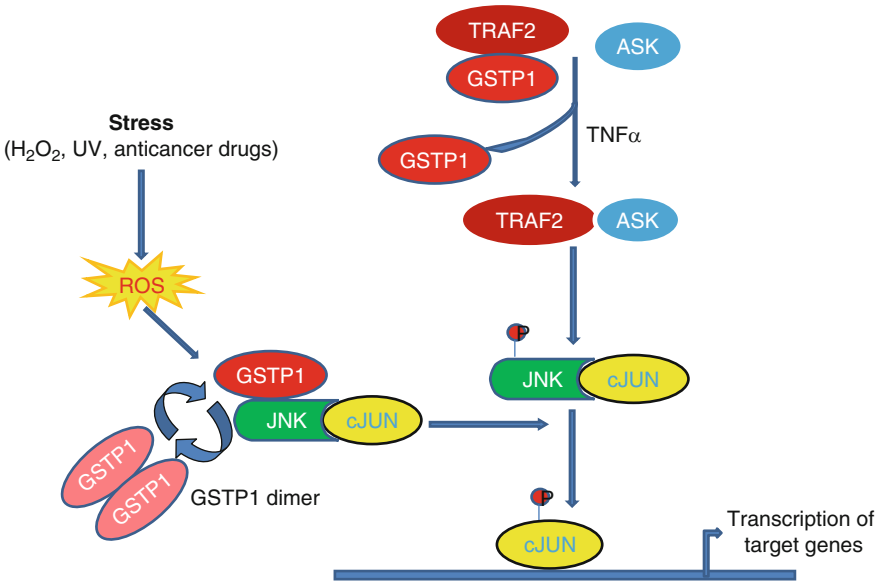

Fig. 13.2 Interaction of GSTP1 with JNK and TRAF2 and modulation of signaling pathways. In normal cells GSTP1 form complex with JNK to maintain low JNK activity. GSTP1 also binds to TRAF2 and prevent TRAF2–ASK1 binding which leads to low JNK activity in normal cells. Exposure of cells to oxidizing agents or TNF-α, GSTP1 is dissociated from these complexes which result in JNK activation and its downstream signaling pathways

c-terminal region failed to bind and inhibit JNK indicating that in the regulation of JNK enzyme activity, both the active center region and the c-terminal region of GSTP1 are important. Allosteric inhibition of GSTP1 activity by the binding of GSH-DXR and its accompanying conformational change result in the release and activation of JNK and induction of apoptosis via the mitochondrial pathway [73].

13.1.6 GSTP1 and Cancer

Overexpression of GSTP1 has been reported in many human tumors and is correlated with advanced stage and aggressiveness of cancer, resistance to therapy and poor survival [74–78]. In contrast, a study on $GSTP^{-/-}$ mice showed that loss of GSTP1 in the early stages of life increases their susceptibility to develop skin papillomas and lung cancer following exposure to carcinogens [79]. Loss of GSTP1 also markedly enhances colon tumorigenesis in Apc^{Min} mice [80]. These studies suggest that GSTP1 possesses tumor suppressor function in normal cells but also impart protection to cancer cells from chemotherapeutic agents and other physical/radiation stress, suggesting the levels of GSTP1 may significantly influence susceptibility to cancer. In addition to its levels, cellular localization of GSTP1 is also an

important factor in carcinogenesis. A study on oral squamous cell carcinoma showed that GSTP1 was localized in the nucleus of cancer cells, whereas in normal cells it is expressed in the cytoplasm [81]. The results of another study involving 61 primary gliomas indicated that high GSTP1 expression in tumor cells and the presence of the GSTP1 protein in tumor cell nuclei are associated with clinically more aggressive gliomas and are strong predictors of poor patient survival [82]. In human colonic cancer HCT8 cells, nuclear GSTP1 prevented H_2O_2-induced DNA damage by scavenging the formation of lipid-peroxide-modified DNA, indicating that nuclear presence of GSTP1 protects cancer cells from oxidative damage and may increase resistance to chemotherapy [83]. The translocation of GSTP1 into the nucleus was induced by H_2O_2 treatment. Edible mushroom lectin, an inhibitor of the nuclear transport of GSTP1, blocked its nuclear transfer and increased the number of tunnel positive cells, indicating the existence of a specific nuclear transport system for GSTP1 [84]. Some recent reports also indicate the presence of GSTP1 in the mitochondria [85].

Loss of GSTP1 expression probably occurs at the initiation of prostatic carcinogenesis, as methylation of GSTP1 promoter is evident in some 5–10 % of proliferative inflammatory atrophy (PIA) lesions and was found in 70 % of high-grade prostatic intraepithelial neoplasia (HGPIN) lesions [86]. Hypermethylation of the GSTP1 regulatory region is a common somatic alteration identified in human prostate cancer and this alteration might result in the loss of GSTP1 expression [87]. In addition, a methyl-CpG-binding domain (MBD) protein has been identified that mediates hypermethylation of the GSTP1 regulatory region [88]. Loss of GSTP1 activity in prostate epithelial cells make them susceptible to genotoxic insult from heterocyclic amine, found in cooked food, predisposing to prostatic carcinogenesis [89]. Silencing of GSTP1 using siRNA approach in normal human prostate epithelial RWPE1 cells caused increased intracellular production of ROS and higher susceptibility of cells to H_2O_2-mediated oxidative stress [90]. This study also demonstrated a significant increase in the levels of 8-oxo-2′-deoxogunosine (8-OHdG), an oxidative DNA damage marker, in prostate adenocarcinomas, compared to benign tissue from the same individuals, which positively correlated with the loss of GSTP1 activity and correlates with GSTP1 promoter hypermethylation [94]. It is hypothesized that reduced GSTP1 activity and/or expression in individuals with allelic variations alter their capacity to detoxify potential carcinogens, which might lead to neoplastic transformation and initiation of prostate cancer [91].

The core function of GSTP1 involves detoxifying xenobiotics including carcinogens via conjugation to GSH [92]. Sustained cellular inhibition of GSTP1 expression and activity may promote stepwise accumulation of genetic damage and cellular transformation necessary for carcinogenesis. Numerous studies on different types of human cancers and GSTP1 polymorphisms have identified that GSTP1 polymorphic forms which possess decreased enzymatic activity are closely associated with cancer development [93]. Another function of GSTP1 which is independent of its enzymatic activity is its role in evading apoptosis by binding to and inhibiting JNK activity. Exposure of tumor cells to chemotherapeutic agent, physical/radiation stress leads to dissociation of GSTP1 from JNK. Free JNK is phosphorylated

by MAPK kinases, and in turn phosphorylates its downstream effectors leading to apoptosis. This provides a explanation for the overexpression of GSTP1 and its association with chemotherapeutic resistance [94]. Studies have shown that levels of GSTs including GSTP1 may elevate in response to selection pressure to anticancer drugs as well GSTP1 is elevated in many cancers as compared to normal tissues. There is convincing evidence that overexpression of GSTP1 in majority of human cancers may lead to resistance to chemotherapy, more so if GSTP1 overexpression is associated with overexpression of multi-drug resistance proteins MRP1 and MRP2 [95]. Consequently, GSTP1 has become an attractive drug target [96].

13.2 Agents Modulating GSTP1 Expression

13.2.1 GSTP1 Inhibitors

Over the last two decades, a great deal of effort has been expended in identifying molecules capable of inhibiting GSTs, in particular GSTP1, in order to modulate tumor cell resistance to anticancer agents. One such agent is a naturally occurring polyphenolic compound belonging to the family of tannins, known as thonningianin, which is isolated from the roots of the African medicinal herb, thonningianin sanguine. This agent was reported to be a potent *in vivo* inhibitor of GSTs in rat liver and GSTP1 in humans [97]. Gossypol, a polyphenol derived from cotton seeds was reported to be a potent inhibitor of GSTP-alpha and -pi isozyme activity with IC_{50} values of 1.6 and 7.0 μM, respectively, in human carcinoma cell lines of breast (MCF-7, T47-D), ovarian (OVCAR-3) colon (HCT-8), and pancreatic (MiaPaCa) cancer [98]. Studies on quercetin have shown that GSTP1 enzyme activity was inhibited completely after 1 h incubation with 100 μM quercetin or 2 h incubation with 25 μM quercetin, and even doses of quercetin between 1 and 10 μM inhibit GSTP1 activity to a significant extent, showing that quercetin effectively inhibits human GSTP1 in a time and dose dependent manner [99]. The inactivation mechanism involves most likely the covalent modification of cysteine 47 in GSTP1 by quercetin quinone or its quinone methides [99]. Several excellent reviews are available in the literature and extended discussion is beyond the scope of this chapter.

13.2.2 GSTP1 Inducers

Epigenetic modifications are commonly observed and occur consistently in various human cancers. The key processes responsible for epigenetic regulation are DNA methylation, modifications in chromatin (covalent modification of core histones), nucleosome positioning (physical alteration), and posttranscriptional gene regulation by noncoding RNA (micro-RNAs). The most interesting and important feature of epigenetics in disease development is the fact that unlike genetic changes,

epigenetic alterations can be modified by the environment, diet or pharmacological intervention. Attempts to therapeutically reverse epigenetic gene silencing in cancer has been attempted with the Food and Drug Administration (FDA) approved inhibitors of DNA methyltransferases such as azacytidine, decitabine, zebularine, procainamide and clinical development of HDAC inhibitors such as vorinostat, MS-275, valproic acid and some others have provided limited efficacy and increase occurrence of side effects [100].

Dietary phytochemicals present in fruits, vegetables and beverages have been shown to possess potential anticancer properties. There has been considerable interest in the use of naturally occurring phytochemicals for cancer prevention. Accumulated evidence demonstrates that phytochemicals can work through a number of complementary and overlapping mechanisms of action, including induction of detoxification enzymes, antioxidant effects, inhibition of the formation of nitrosamines, binding/dilution of carcinogens in the digestive tract, alteration of hormone metabolism and modulation of carcinogenic cellular and signaling events [101]. Studies in the past decade demonstrate that phytochemicals can influence the activity of various epigenetic factors, such as DNMTs and HDACs, and may be useful to prevent and treat various diseases, including cancer. Although several dietary agents or nutrients regulate different molecular and epigenetic targets in human cancers, this chapter focuses on the role of bioactive dietary phytochemicals as GSTP1 inducers in prostate cancer. These include various plant flavonoids which are part of a family of naturally occurring polyphenolic compounds representing one of the most prevalent classes of compounds. These polyphenolic compounds are ubiquitously present in vegetables, nuts, fruits, beverages and medical herbs and include flavones, flavonols, flavanones, flavanols, anthocyanins and isoflavones [102]. More than 8,000 compounds with flavonoid structure have been identified and their average consumption by humans varies widely. Flavonoids exert a wide range of biochemical and pharmacological properties, with one of the most investigated effects being their cancer preventive activities [103]. The cancer protective effects of flavonoids have been attributed to a wide variety of mechanisms, including free radical scavenging, modifying enzymes that activate or detoxify carcinogens, and inhibiting the induction of the transcription factor AP1 activity by tumor promoters [101].

Green tea, the most consumed beverage in the world has been widely reported to possess anticancer properties due to the presence of significant amount of catechins *viz*. epicatechin (EC), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG), present therein. EGCG is the most abundant constituent and has been found to have significant anti-proliferative and anti-tumor effects both in *in vitro* and in animal cancer models [104]. Green tea catechins may contribute to *in vivo* efficacy by a number of ways, i.e. apoptosis induction, cell cycle arrest etc. but are also found to affect the levels and activity of various phase II enzymes, including GSTP1 in both *in vivo* and *in vitro* studies [105]. In a study on precancerous liver lesions in Wistar rats established by multiple low-dosage N-nitrosodiethylamine (NDEA) injections, followed by intraperitoneal CCl_4 injection and partial hepatectomy (PH), oral feeding of 0.1 % tea polyphenols in drinking water for 8 weeks decreased the number and area of GSTP1-positive foci which

were over-expressed in the NDEA-CCl_4-PH-treated rats compared to the control group. mRNA and protein expression of GSTP1 was also found to be increased significantly in the NDEA-CCl_4-PH-treated group which also decreased after polyphenols treatment, suggesting that tea polyphenols prevent the occurrence and progression of precancerous liver lesions in rats [106]. Green tea polyphenol extract (GTP) stimulated the transcription of Phase II detoxifying enzymes through the ARE in human hepatoma HepG2 cells and demonstrate that the stimulation MAPKs may be the potential signaling pathways utilized by GTP to activate ARE-dependent genes. EGCG potently induced ARE-mediated gene expression, activated MAP kinase pathway, stimulated caspase-3 activity, and induced apoptosis in human hepatoma HepG2-C8 cells [107]. In *in-vitro* exposure of human prostate cancer LNCaP cells to GTP (1–10 μg/ml) up to 7 days caused a dose- and time- dependent re-expression of GSTP1. This re-expression correlated with DNMT1 inhibition and extensive demethylation in the proximal GSTP1 promoter and regions distal to the transcription factor binding sites. This study also demonstrated that GTP treated cells had reduced MBD2 association with accessible Sp1 binding sites leading to increased binding and transcriptional activation of the GSTP1 gene [108]. Furthermore, re-expression of GSTP1 after GTP exposure protected the cells from H_2O_2-mediated DNA damage through decreased ROS production suggesting that GSTP1 may be an important target for primary prevention of prostate cancer [90]. In female rats, long-term ingestion of green tea extracts increases cytosolic GST activity [109]. A trial of 4 weeks of Polyphenon E administration to healthy subjects resulted in differential effects on GST activity and level based on baseline enzyme activity/level, with GST activity and GSTP1 level increased significantly in individuals with low baseline enzyme activity/level, demonstrating that green tea polyphenols act as GSTP1 inducers [110].

Sulfur-containing phytochemicals known as isothiocyanates occur naturally as glucosinolate conjugates in cruciferous vegetables such as broccoli, cauliflower, cabbage, radish etc. [111]. These include methylsulfinylalkyl isothiocyanates like sulforaphane (SFN) and aromatic isothiocyanates, phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC). Isothiocyanates exert chemoprotective effects partly by inducing phase II enzyme, thus enhancing the elimination of activated carcinogens. SFN has been shown to increase GSTP1 and other phase II enzymes expression, at mRNA, protein and activity levels in cell culture study utilizing human prostate cancer cells [112, 113]. A study using breast cancer MCF7 cells suggested that the regulatory effects of MRP1 and GSTP1 expression on SFN-dependent induction of phase II genes are ultimately mediated by altering nuclear Nrf2 levels [114]. SFN was also found to increase GSTP1 levels in the liver, forestomach, glandular stomach, and mucosa of proximal small intestine, and to a lesser degree in the lungs of mice after 15 μM SFN/mouse per day for 5 day treatment [115]. A study conducted to investigate the structure-activity relationships of four arylalkyl-isothiocyanates *viz.* (4-phenylbutyl isothiocyanate (PBITC), 6-phenylhexyl isothiocyanate (PHITC), benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC) for their inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) oxidation and effects on xenobiotic-metabolizing enzymes in rats and mice.

Overall, PEITC was more potent than BITC but less potent than PBITC and PHITC. Although all four isothiocyanates extensively inhibited NNK oxidation in rat lung and nasal mucosa microsomes as well as mouse lung microsomes *in vitro*. PEITC (IC_{50} of 120–300nM) was found to be more potent than BITC (IC_{50} of 500–1400nM) but less potent than PBITC and PHITC (IC_{50} of 15–180nM) and could protect against a broad spectrum of carcinogens and potential toxic agents [116]. In addition, BITC was shown to significantly induce GSTP1 activity in rat liver epithelial RL34 cells [117]. In a population based case-control study, intake of cruciferous vegetable consistent with high isothiocyanate exposure reduced breast cancer risk and also ameliorate the effects of the *GSTP1* genotype [118]. In another study, PEITC was found to be effective in inhibiting the development of prostate tumors in TRAMP mice. The effects of dietary PEITC on DNMT1 expression in a cyproterone and testosterone-primed Wistar rat was analyzed and it was found that both the enhancement of DNMT1 and proliferation was inhibited by feeding 5 mg/kg PEITC [119]. Cruciferous vegetables, including brussels sprouts and cabbage, also contain another class of chemoprotective compounds known as dithiolethiones. A compound representative of this group -3*H*-1,2-dithiole-3-thione (D3T) has been extensively studied as a chemoprotective agent, and synthetic substituted dithiolethiones, including oltipraz (5-[2-pyrazinyl]-4-methyl-1,2-dithiol-3-thione), and anethole dithiolethione (ADT; 5-[4-methoxyphenyl]-1,2-dithiole-3-thione) have been developed for pharmaceutical applications for their antioxidant, chemotherapeutic, radioprotective, and chemopreventive properties [120]. Dithiolethiones act *via* some alternate mechanisms including increased expression or activity of phase II enzymes together with GSTP1[121]. D3T and oltipraz increased glutathione and phase II enzyme levels in several organs of rat [122].

Curcumin, an antioxidant isolated from turmeric has been shown to attenuate chemical carcinogenesis in rodents [123]. Curcumin has been shown to reduce the expression of GSTP1 in human leukemia cell lines and inhibition of GSTP1 catalytical activity indicate curcumin inhibits GSTP1 irreversibly mainly by binding to cysteine residues of GSTP1, especially Cys-47 [124]. Curcumin was well tolerated at doses up to 8 g per day in a phase I study in patients with pre-malignant conditions [125, 126].

13.3 Conclusions and Future Directions

GSTP1 is a caretaker of both normal and tumor cells and provides protection from xenobiotics and other forms of cellular stress. The association of high levels of GSTP1 with malignant disease and drug resistant cancers highlights its ability to participate in detoxification process. Additionally, elevated serum GSTP1 is used as a serum tumor marker in predicting effectiveness of cancer to chemotherapy. This suggests that GSTP1 can be exploited as an attractive therapeutic target either to suppress its activity to overcome drug resistance in malignant cells; or to induce GSTP1 to protect from genotoxic and other forms of stress in normal cells. Epigenetic

silencing of GSTP1 gene is frequently observed in early stage cancer development where inhibitors of DNA methyltransferase and HDACs lead to GSTP1 gene re-expression. Because of the side-effects associated with these epigenetic agents they have limited scope in the prevention and therapy of cancer. Natural dietary agents may therefore be developed as safe and effective replacement, as in recent years dietary polyphenols have been reported to reverse epigenetic modifications at various levels. Therefore, identification and development of dietary polyphenols could be an effective approach in protecting normal cells from oxidative stress, through GSTP1 induction, and prevent its neoplastic transformation and malignant progression which remains a better approach to cancer prevention.

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Chapter 14
Epigenetic Regulations of mRNAs and miRNAs by Nutraceuticals

Yiwei Li, Dejuan Kong, Aamir Ahmad, Bin Bao, and Fazlul H. Sarkar

Abstract Both genetic alterations and epigenetic regulations of genes could lead to the development of human cancers. However, recent studies have shown that epigenetic alteration contributes significantly not only to the development of cancer but also responsible for the progression of cancer to metastatic disease. The epigenetic regulations of specific genes in human cancer cells include DNA methylation, acetylation, histone modification, nucleosome remodeling, and small non-coding RNA regulation including the regulation of microRNAs (miRNAs). Among many epigenetic regulations, DNA methylation is the most common event and has been well studied for understanding the mechanisms of epigenetic regulation of genes. The DNA hypermethylation occurs in the promoter sequences of tumor suppressor gene or tumor suppressive miRNAs leading to the down-regulation in the expression of tumor suppressor mRNAs or miRNAs, resulting in the development and progression of various cancers. Interestingly, recent studies have shown that several non-toxic natural agents known as nutraceuticals including isoflavone, curcumin, (−)-epigallocatechin-3-gallate, resveratrol, indole-3-carbinol, 3,3′-diindolylmethane, and lycopene could demethylate DNA at their hypermethylation sites or modulate histone, demonstrating their potential roles in the epigenetic regulation of mRNAs and miRNAs. These epigenetic regulations of mRNAs and miRNAs could be one of the molecular mechanisms by which nutraceuticals inhibit carcinogenesis and cancer progression, and thus either nutraceuticals or their synthetic analogs could serve as novel demethylating agents for the treatment of human malignancies.

Y. Li • D. Kong • A. Ahmad • B. Bao • F.H. Sarkar (✉)
Department of Pathology, Barbara Ann Karmanos Cancer Institute,
740 Hudson Webber Cancer Research Center, Wayne State University
School of Medicine, 4100 John R, Detroit, MI 48201, USA
e-mail: fsarkar@med.wayne.edu

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14.1 Introduction

Human cancer is the second leading cause of death after cardiovascular disease in the United States and in the world. It is known that both genetic alterations and epigenetic regulations of genes could cause human cancers. The genetic changes including DNA point mutations, gene amplification, gene translocation, etc. have been traditionally believed as major causes of cancer development. However, recent studies have demonstrated that epigenetic alterations contributes significantly to the development and progression of cancers [1]. Moreover, it has been found that genetic and epigenetic regulations are not separate biological events in cancer. Epigenetic regulations could cause genetic mutations while genetic mutations in epigenetic regulators could alter epigenome [1], suggesting the complex biological regulations of these genetic events in the development and progression of cancer.

The epigenetic regulation of specific genes in human cancer cells include DNA methylation, histone modification, nucleosome remodeling, and small non-coding RNA (ncRNA) regulation including microRNAs (miRNAs). These regulations lead to the alterations in the expression of genes without altering the DNA sequences. Among the different types of epigenetic regulations, DNA methylation is the most common event and has been well studied. DNA methylation is heritable and plays critical role in cell differentiation and embryogenesis. However, the hypermethylation occurs in the DNA sequences in the promoter of tumor suppressor genes which could cause gene silencing through the obstruction of transcriptional activators, leading to the development and progression of various cancers (Fig. 14.1).

In recent years, studies have focused on the investigations of the roles and the epigenetic regulation of miRNAs in cancer development and progression. The miRNAs could inhibit its target gene expression by binding to the 3′-untranslated region (3′-UTR) of target mRNA, causing either mRNA degradation or inhibition of translation. The miRNAs could be oncogenic or tumor suppressive depending on their specific functions during cancer development and progression. Interestingly, it has been found that some miRNAs are also epigenetically regulated in various cancers [2], resulting in altered expression of miRNAs and their target mRNAs. The DNA hypermethylation occurs in the promoter region of miRNA gene which could result in the low expression of miRNAs and, in turn, up-regulates the expression of specific target mRNAs and proteins. The epigenetically regulated tumor suppressive miRNAs could cause increased expression of oncogenes both at the mRNA and

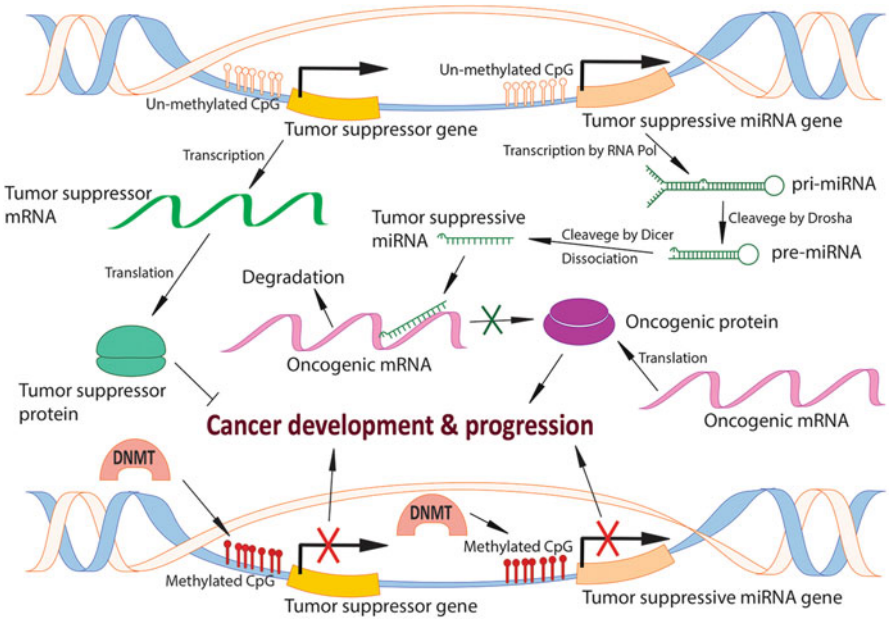

Fig. 14.1 DNA methylation regulated mRNA and miRNA expressions in cancer development and progression

protein levels, which in part could be responsible for the development and progression of various cancers (Fig. 14.1).

Since epigenetic regulations of mRNAs and miRNAs through DNA methylation and histone modification play important roles in cancer development and progression, targeting the epigenetic deregulations in cancers could become a novel and effective approach to fight the battle against cancers. Several epigenetic inhibitors have been synthesized and used in epigenetic therapy trials to re-express abnormally silenced tumor suppressor genes. However, the side-effects of the demethylating agents and histone deacetylase inhibitors (HDAC inhibitors) appear side-by-side with the beneficial effects [3]. Interestingly, recent studies have shown that several non-toxic natural agents known as nutraceuticals including isoflavone, curcumin, (−)-epigallocatechin-3-gallate (EGCG), resveratrol, indole-3-carbinol (I3C), 3,3′-diindolylmethane (DIM), and lycopene could demethylate DNA sequences or inhibit HDACs, demonstrating their roles in epigenetic regulation of mRNAs and miRNAs. These epigenetic regulations of mRNAs and miRNAs could be one of the molecular mechanisms by which nutraceuticals inhibit carcinogenesis and cancer progression, suggesting that either nutraceuticals or their synthetic analogs could serve as novel demethylating agents for the treatment of human malignancies.

14.2 Epigenetic Regulation of mRNAs in Cancers

Epigenetics refers to heritable as well as non-heritable changes in gene expression and cellular phenotype that are not due to alterations in DNA sequence. Epigenetic regulations could alter the expression of mRNA of specific genes. During cancer development and progression, the epigenome precedes multiple alterations including genome-wide loss of DNA methylation (known as hypomethylation), frequently increased methylation of CpG islands in the gene-specific promoter sequence, changes in histone modification and nucleosome, and alterations in ncRNA profile. These alterations are beginning to be appreciated as the molecular basis of carcinogenesis and cancer aggressiveness. Therefore there are significant efforts in the areas of drug development research focusing on epigenetic deregulation of genes for the treatment of human malignancies.

14.2.1 DNA Methylation

Among the epigenetic regulations, DNA methylation is the most widely investigated area in cancer research. During the process of DNA methylation, methyl group is added to cytosine base of CpG dinucleotides through enzymatic methyl transfer catalyzed by DNA methyltransferases (DNMTs). DNA methyltransferases consist of DNMT1, DNMT3A, DNMT3B, and DNMT3L, which are grouped into maintenance and *de novo* methyltransferases. DNMT1 is a maintenance methyltransferase, which recognizes hemimethylated DNA produced during cell division and methylates newly synthesized CpG dinucleotides, to maintain the status of methylation. DNMT3A and DNMT3B are *de novo* methyltransferases to produce DNA methylation during embryogenesis or tumorigenesis. DNMT3L does not possess enzymatic activity; however, it regulates the activity of other methyltransferases to alter the status of methylation.

DNA methylation is a fundamental event in epigenetic regulation, and plays critical roles in the control of gene expression. The methylation of CpG islands, which are the regions with a high density of CpG dinucleotide, in the promoters of genes obstructs transcriptional activators, leading to the down-regulation of mRNA expression. In addition, DNA methylation also influences the remodeling of nucleosome. Wrapped nucleosomal DNA is less accessible than linker DNA; therefore, compressed nucleosomes strongly prevent transcription activators binding to DNA sequences. The methylation of CpG islands allows compressed nucleosome formation and blocks transcription. Moreover, DNA methylation also provides an environment for several methyl-CpG binding proteins including MBD1, MBD2, MBD3, and MECP2, which recruit histone-modifying enzymes to modify histone and regulate gene expression. Therefore, DNA methylation together with other epigenetic regulations could lead to the aberrant expression of tumor suppressor genes, causing carcinogenesis and cancer progression.

14.2.2 DNA Hypermethylations in the Promoters of Tumor Suppressor Genes in Cancers

In normal cell, about 50 % of the CpG islands in the promoter region of genes are un-methylated and these genes are expressed for normal functions [4]. In cancer cells, more methylations occur within CpG islands of promoters, especially hypermethylations in the promoter region of tumor suppressor genes. It has been found that 5–10 % of normally unmethylated CpG islands in the promoter regions become highly methylated in various human cancer [5]. DNA hypermethylation has been commonly correlated with significant down-regulation of gene expression. The reported gene silencing due to hypermethylation in cancers include hMLH1, APC, E-cadherin, CHFR, CASP8, TGF-βRII, p73, HOX A11, COMT, SPRY2, RASSF1A, GPR54, CDH1, RSK4, etc. These DNA hypermethylations commonly do not appear in normal cells; however, it could be observed in hyperplasia, pre-cancerous cells, and in cancer cells.

It is now well known that APC is a tumor suppressor gene. The DNA hypermethylation in the APC gene promoter has been found in atypical hyperplasia, early pre-cancerous cells, and in cancer cells. The frequency of DNA hypermethylation in the APC promoter region has been shown to be negatively correlated with progression of some types of cancer, suggesting that APC hypermethylation could be an early event in tumorigenesis [6]. SPRY2 is another tumor suppressor gene involved in the control of cell proliferation, differentiation and angiogenesis through the inhibition of MAPK signaling. The expression of SPRY2 has been shown to be down-regulated in various cancers because of the DNA hypermethylation in SPRY2 promoter [7]. RASSF1A is also a tumor suppressor gene which inhibits RAS-MAPK signaling. It has been found that the RASSF1A promoter is hypermethylated in cancer cells, leading to reduced expression of RASSF1A consistent with malignant transformation of different types of cells [8]. In addition, more DNA hypermethylations have been observed in the promoters of other tumor suppressor genes in various cancers [9], demonstrating that the silencing of tumor suppressor genes is in part regulated through epigenetics in human cancers.

14.2.3 Histone Modifications in the Regulation of Gene Expression

It is well known that highly conserved histone proteins (such as H1, H2A, H2B, H3 and H4) and DNAs are the basic components of eukaryotic chromatin. The histones undergo a series of post-translational modifications including acetylation, methylation, phosphorylation, ADP-ribosylation, and ubiquitination. Among them, acetylation and methylation of histones are more relevant to the regulation of gene expression (Fig. 14.2). Histone acetylation has been widely investigated and believed to be one of the important modifications during cancer development. It has been found

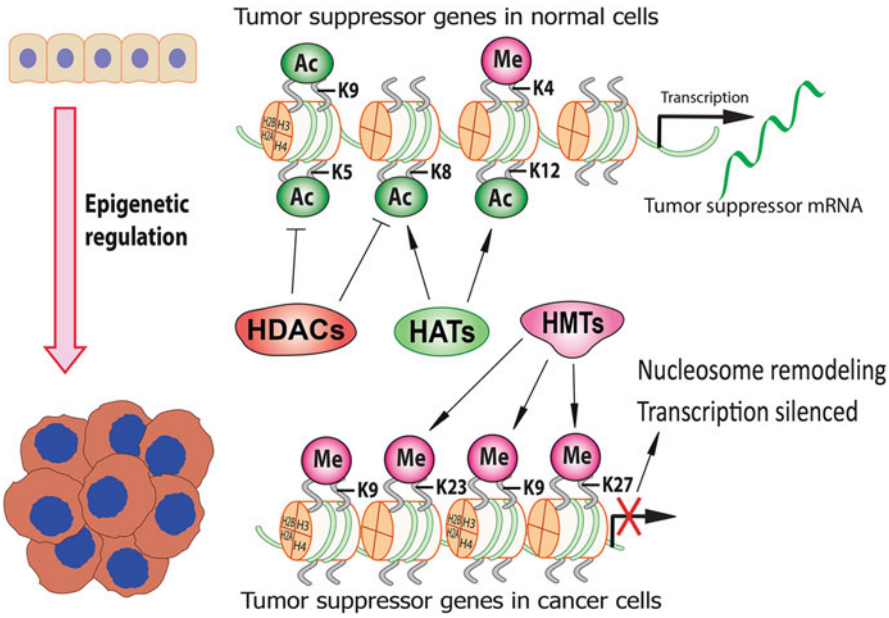

Fig. 14.2 Histone modifications in cancer development

that some selected lysines (such as lysines 9 and 12) are acetylated by histone acetyltransferases (HATs) or deacetylated by histone deacetylases (HDACs) to keep the balance of stable status in the DNA modification [10]. Methylation of lysine residues in histone is another important post-translational modification involved in cancer development. It has been known that methylation of H3 at different lysines could lead to deregulation in the expression of genes. The methylation of H3 at lysine 4 (H3K4) could activate gene expression while the methylation of H3 at lysine 9 (H3K9) and 27 (H3K27) could inhibit the expression of genes [10, 11].

Although both DNA methylation and histone modification have their own enzymes to catalyze different chemical reaction, DNA methylation and specific histone modifications could influence each other to regulate gene expression. Histone methylation could influence DNA methylation to form different methylation patterns whereas DNA methylation could serve as a template for some histone modifications [11]. The molecular interactions among histone, DNA methyltransferases, and other enzymes and proteins contribute to orchestrate interrelationship between DNA methylation and histone modifications. During DNA methylation, methylation-binding proteins (MBDs) and histone deacetylase (HDAC) are recruited to the chromosome. MBDs prevent transcription factors and cofactors binding to the promoter of genes, and thus, inhibit the expression of genes. HDAC in the region of methylated DNA also reduces the activity of the promoter and deacetylates the lysine of histone, resulting in the tightly packed chromosomes which block transcription factor access [1, 11]. Therefore, both DNA methylation and histone deacetylation

work together in regulating gene expression, which prompted the development of drugs that could function as demethylating agents of the inhibitors of HDACs. Advances have been made in the clinical arena for testing the anti-tumor activity of some agents; however, more clinical trials especially phase III clinical trials are warranted.

14.3 Epigenetic Regulations of miRNA Expression in Cancers

The miRNA is one type of short noncoding RNA that down-regulates the expression of its target genes though degradation of target mRNA or interruption of target translation. Emerging evidences have shown that DNA methylation in the promoter region of miRNA genes could also down-regulate the expression of specific tumor suppressive miRNAs, resulting in the up-regulation of oncogenic targets of these miRNAs (Fig. 14.1). The up-regulated oncogenic signaling caused by DNA methylation mediated through miRNA down-regulation could promote carcinogenesis, cancer invasion and metastasis. In the following section we will summarize the role of selected miRNAs whose expression has been found to the regulated through epigenetic events although we cannot catalog all miRNAs due to space limitation.

DNA hypermethylation in the region of miR-9 promoter has been found in renal, gastric, and lung cancers [12, 13]. The hypermethylation caused the silencing of miR-9 gene resulting in reduced expression of miR-9. Importantly, it has been found that the hypermethylation of the miR-9 promoter is associated with cancer development, metastasis, recurrence, and shorter overall survival [12, 13], suggesting the prognostic value of miR-9 methylation and further suggest that selective demethylating agents would be useful therapeutic approach for these malignancies.

The miR-34 belongs to a tumor-suppressor miRNA family. The expression of miR-34 family could be regulated by tumor suppressor p53 and DNA hypermethylation [14]. The down-regulation of miR-34 expression is commonly observed in various cancers. We and other investigators have found lower expression of miR-34 which was in part due to DNA methylation of the promoter region of miR-34 gene [15, 16]. It has been shown that androgen receptor (AR), Notch-1, and SIRT1 are the direct targets of miR-34. Therefore, the AR, Notch-1, and SIRT1 signaling is usually up-regulated in cancer cells due to the silencing of miR-34 expression, which could be causally linked with cancer development and progression.

The miR-29a is also a tumor suppressive miRNA. We and other investigators have found that miR-29a is down-regulated in lymphoma, prostate and pancreatic cancer cells and tissues due to DNA methylation of the promoter of miR-29a gene [17, 18]. Other studies have shown that miR-29 family directly targets both DNMT3A and DNMT3B and that the down-regulation in the expression of miR-29 family causes overexpression of DNA methyltransferases 3A and 3B [19]. These findings suggest a regulatory loop of miR-29/DNMT/methylation in the epigenetic regulation of cancer specific genes and their signaling.

The miR-124a is a known tumor suppressive miRNA. The DNA hypermethylation and the epigenetic silencing of miR-124a have been observed in different types of cancers [20]. The expression of miR-124a has been found to be significantly down-regulated which leads to the overexpression and activation of its target gene, CDK6. The epigenetic silencing of miR-124a expression also leads to the phosphorylation of tumor suppressor gene retinoblastoma [20], resulting in the promotion of cancer cell growth.

The miR-129-2 is another miRNA with tumor suppressor feature. The miR-129-2 directly targets the expression of SOX4 by 3′-UTR binding. It has been found that the level of miR-129-2 was significantly down-regulated while the expression of SOX4 was highly up-regulated in gastric and endometrial cancer cells [21]. Moreover, the DNA hypermethylation in the miR-129-2 CpG islands was observed in gastric and endometrial cancer cell lines and in 68 % of human endometrial cancer tissues. Histone acetylation and DNA demethylation has been shown to up-regulate the expression of miR-129-2, and consequently down-regulates the expression of SOX4, resulting in the inhibition of cancer cell proliferation [21], suggesting the epigenetic regulation of miR-129-2 in cancers.

The down-regulation of tumor suppressive miR-145 has been observed in various cancers. It has been found that the expression of miR-145 is silenced through DNA hypermethylation and p53 mutation. Moreover, the promoter region of miR-145 gene has been found to be highly methylated in both human prostate cancer tissues and cell lines [22]. Since miR-145 could down-regulate OCT, SOX2 and KLF4 which are markers of the embryonic stem cells, the epigenetic deregulation of miR-145 in cancers could contribute to the growth of cancer stem cells; however, further studies in this area is required.

The miR-152 is also a tumor suppressive miRNA which could be deregulated by DNA hypermethylation. The methylation of miR-152 promoter and low expression of miR-152 has been observed in acute lymphocytic leukemia, endometrial and other cancers [23]. The expression of miR-152 could be recovered by demethylating agent 5-aza-dC. It has been found that DNMT1, E2F3, and MET are targets of miR-152. The methylation of miR-152 promoter could increase the expression of DNMT1, E2F3, and MET [23], leading to high methylation status during cancer development.

The miR-200 family has been known to play important roles in the regulation of epithelial-to-mesenchymal transition (EMT) through the inhibition of ZEB1 and ZEB2. ZEB1 and ZEB1 are the transcriptional repressors of E-cadherin, which is a critical molecule for epithelial structure. The DNA hypermethylation in the region of miR-200 promoter has been found in lung and bladder cancers [24]. The methylation of miR-200 promoter caused lower expression of miR-200, leading to EMT and increased proliferation of cancer cells [24].

In addition, the epigenetic deregulation of other miRNAs including miR-92, miR-127, miR-137 miR-148a, miR-203, miR-26, etc. have also been observed in different types of cancers [2], which leads to the development and progression

of cancers. Therefore, targeting aberrant miRNA expression altered by epigenetic regulation could be an effective strategy for cancer prevention and treatment. Although there have been some progress in the areas of drug development such as demethylating agents or HDAC inhibitors, there remains many challenges especially the unwanted toxicity of these agents, which prompted many investigators to turn into agents that are abundantly found in the nature and are known to be non-toxic as discussed in the following paragraphs.

14.4 Epigenetic Regulations of mRNAs and miRNAs by Nutraceuticals

Emerging evidences have demonstrated that several nutraceuticals including isoflavone, curcumin, EGCG, resveratrol, and lycopene could serve as epigenetic regulators to reverse the deregulated expression of tumor suppressive mRNAs and miRNAs, leading to the inhibition of cancer development and progression (Fig. 14.3). The effects of these selected agents are discussed below although we cannot summarize all natural agents because of space limitation.

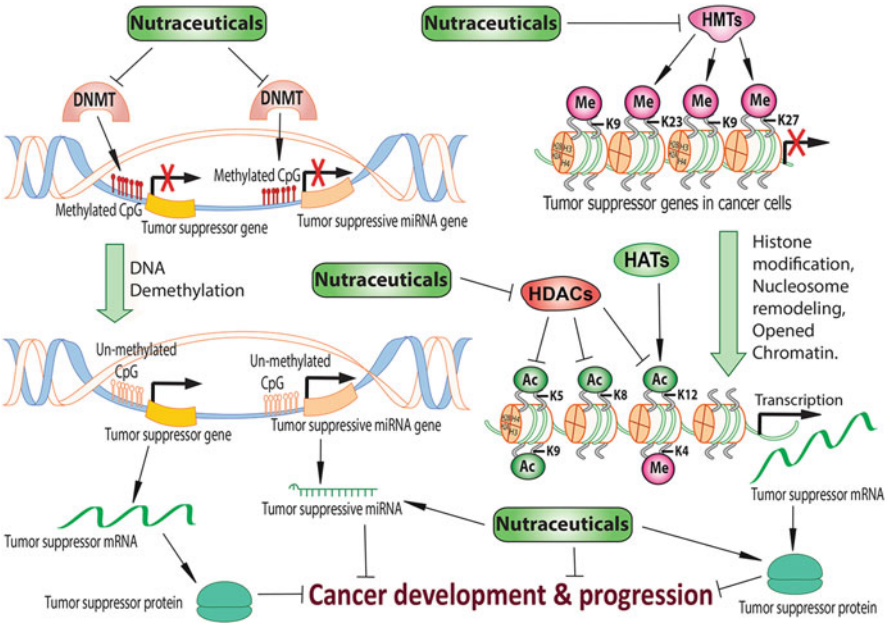

Fig. 14.3 The effects of nutraceuticals on epigenetic regulations in cancer development and progression

14.4.1 Epigenetic Regulations by Isoflavone

Isoflavones are mainly derived from soybean and could epigenetically up-regulate the expression of tumor suppressor mRNAs and miRNAs by modulating DNA methylation and chromatin configuration, leading to the suppression of cancer cell survival. To explore the effect of isoflavone genistein on epigenetic regulation of miRNAs, the miRNA expression profiles of PC-3, DU145, and LNCaP prostate cancer cells after genistein and Aza-dC treatment were compared. It has been found that genistein had similar effects on miRNA regulation compared to Aza-dC, suggesting that genistein and demethylating agent Aza-dC could have similar epigenetic regulatory effects on miRNAs [25] which is in part due to the role of genistein as a demethylating agents among many other effects of genistein. We have also found higher level of methylation in the promoter region of miR-29a and miR-1256 in prostate cancer cells compared to normal prostate epithelial cells [17]. Importantly, we found that isoflavone could demethylate the methylated promoter of miR-29a and miR-1256 and, in turn, up-regulate the expression of miR-29a and miR-1256. By up-regulation of miR-29a and miR-1256, isoflavone could reduce the expression of TRIM68 and PGK-1, which are targets of miR-29a and miR-1256. However, it is important to note that isoflavone was not a pan-demethylating agent like Aza-dC. We found that Aza-dC up-regulated oncogenic miR-155 and miR-421 expression by demethylation while isoflavone decreased the expression of miR-155 and miR-421, suggesting the specific targeting effect of isoflavone [17]. Other investigators also reported that isoflavone genistein could regulate the expression of miR-145, miR-221, and miR-222, leading to the inhibition of prostate cancer growth through epigenetic regulations [26, 27].

Studies have shown in LNCaP and DuPro prostate cancer cells that isoflavone genistein could up-regulate the expression of tumor suppressor genes $p21^{WAF1}$ and $p16^{INK4a}$. This effect of isoflavone genistein was mediated by epigenetic regulation. It has been found that genistein increased the expression of histone acetyltransferases and the level of acetylated histones 3, 4, and H3K4 at the transcription start sites of $p21^{WAF1}$ and $p16^{INK4a}$, leading to the up-regulation of tumor suppressor genes $p21^{WAF1}$ and $p16^{INK4a}$ [28]. In $ARCaP_E$ and $ARCaP_M$ prostate cancer model of EMT, isoflavone genistein affected histone H3K9 acetylation and increased the expression of histone acetyltransferase 1 (HAT1). Moreover, genistein combined with histone deacteylase inhibitor vorinostat could significantly enhance cell death in prostate cancer cells [29]. The effects of isoflavones, genistein and daidzein, on DNA methylations in the promoter regions of glutathione S-transferase P1 (GSTP1) and ephrin B2 (EPHB2) genes have also been tested in prostate cancer cells. After treatment with isoflavones, the authors have found significant demethylation of GSTP1 and EPHB2 promoters with corresponding increase in their protein expression [30]. All these findings demonstrate the potent effects of isoflavone on epigenetic regulations of genes in prostate cancer, and as such genistein may serve as a demethylating agent for the treatment of human malignancies although further in-depth investigations are required.

The effects of isoflavone on breast cancer in terms of epigenetic regulation have also been investigated. A study showed that the promoters of BRCA1 and BRCA2 tumor suppressor genes were highly methylated in MCF-7 and MDA-MB-231 breast cancer cells [31]. However, demethylation agent Aza-dC or isoflavones including genistein and daidzein could reduce DNA hypermethylation and consequently up-regulate the expression of BRCA1 and BRCA2, suggesting the demethylating effect of genistein and daidzein in breast cancer [31]. In MCF-7 breast cancer cells, isoflavone genistein also showed its ability to inhibit the expression of hTERT (human telomerase reverse transcriptase) and DNA methyltransferases (DNMT1, 3a and 3b). Moreover, isoflavone genistein could remodel chromatin structures of the hTERT promoter by induction of trimethyl-H3K9 and reduction of dimethyl-H3K4 in the hTERT promoter. The combination treatment with isoflavone genistein and demethylating agent Aza-dC led to a significant inhibition in the expression of hTERT, suggesting the epigenetic regulation of telomerase by isoflavone genistein [32]. In addition, lignans as isoflavones are one of the major classes of phytoestrogens. The nordihydroguaiaretic acid (NDGA) is a member of the lignan family and it was found that NDGA could reverse DNA hypermethylation in $p16^{INK4a}$ CpG islands and restore its expression in T47D breast cancer cells, leading to cell cycle arrest at G1 phase [33]. These findings suggest the effects of isoflavone on epigenetic regulations in breast cancer and similar effects may occur in other cancers.

BTG3 is a tumor suppressor gene and its expression has been found to be down-regulated in renal cancers due to DNA hypermethylation in the BTG promoter. However, isoflavone genistein and demethylating agent Aza-dC significantly inhibited the DNA hypermethylation in the BTG promoter [34]. Isoflavone genistein and Aza-dC also induced acetylated histones 3, 4, 2H3K4, 3H3K4 and RNA polymerase II at the BTG3 promoter. Moreover, genistein and Aza-dC decreased DNA methyltransferase and methyl-CpG-binding domain 2 activity, leading to increased BTG expression and cell cycle arrest. Similar effects of isoflavone genistein have also been observed in prostate cancer cells [35], suggesting the epigenetic effects of isoflavone on tumor suppressor BTG3 expression and cancer cell proliferation.

In myeloid and lymphoid leukemia, genistein exerted its anti-tumor activity through reactivation of tumor suppressor genes which are commonly silenced by DNA methylation [36]. In the clinical setting, Aza-dC has been used for the treatment of leukemia. It has been found that isoflavone genistein combined with Aza-dC could significantly enhance anti-leukemic activity against murine Aza-dC resistant cells [37], suggesting that genistein could increase the clinical efficacy of Aza-dC through epigenetic regulation. In esophageal squamous cell carcinoma cells, genistein has been shown to inhibit DNA methyltransferase activity and, in turn, up-regulate RARβ, $p16^{INK4a}$, and MGMT expression, causing the inhibition of cancer cell growth [38].

DKK1 is an antagonist of Wnt signaling and DNA methylation in DKK1 promoter has been found in colon cancer cells. The effects of isoflavone genistein on epigenetic regulation of DKK1 have been detected. DNA methylation at the DKK1 promoter was not altered by genistein treatment; however, genistein induced histone

H3 acetylation of the DKK1 promoter region in colon cancer cells, leading to increased expression of DKK1 [39]. These results suggest the epigenetic regulatory effects of isoflavone on Wnt signaling. In addition, *in vivo* animal studies showed that isoflavone exerted its inhibitory effects on DNA methylation. The overall methylation was found to be increased in liver and muscle tissues when monkeys switched from soy diets to no soy diets. The involved genes in epigenetic regulation by isoflavone *in vivo* are specifically homeobox genes (HOXA5, HOXA11, and HOXB1) and ABCG5 [40]. These reported results all support the epigenetic effects of isoflavone although further mechanistic and clinical studies are warranted.

14.4.2 Epigenetic Regulations by Curcumin

Curcumin is a natural compound present in turmeric and possesses anti-inflammatory, antioxidant, and anti-cancer activity. Experimental studies have demonstrated that curcumin could mediate epigenetic modulation of miRNA expression. The miR-203 is a tumor suppressive miRNA and it is frequently down-regulated in bladder cancer because of DNA hypermethylation in its promoter [41]. Curcumin could up-regulate the expression of tumor suppressive miR-203 in bladder cancer through demethylation of miR-203 promoter. Since Akt2 and Src are the targets of miR-203, the up-regulation of miR-203 by curcumin could down-regulate the expression of Akt2 and Src, leading to reduced proliferation and increased apoptosis of bladder cancer cells [41], suggesting the epigenetic regulatory effects of curcumin on miRNA expression.

In addition to the regulation of miRNA, curcumin could also epigenetically regulate mRNA expression, leading to cell growth inhibition. Curcumin has been found to inhibit the activities of DNMT, HAT, and HDAC. However, the molecular mechanism by which curcumin inhibits DNMT is unclear. By molecular docking analysis of curcumin and DNMT1 interaction, it was found that curcumin could block the catalytic thiolate of C1226 of DNMT1 to inhibit the activity of DNMT1 [42]. Another mechanism of DNMT inhibition by curcumin involves cyclic nucleotide phosphodiesterases (PDEs). The effects of curcumin on PDE-regulated DNMT1 have been investigated in B16F10 murine melanoma cells. It has been found that curcumin was able to down-regulate PDE1 and PDE4 activities and, in turn, inhibited the expression of DNMT1, leading to the inhibition of melanoma cell proliferation [43]. Histone deacetylase inhibitors have been used as epigenetic drugs but have shown low efficacy in cancer monotherapy. It was found that HDAC inhibitors could activate tumor-progressive genes to enhance cell migration and tumor metastasis. However, HDAC inhibitors combined with curcumin have been shown to suppress HDAC inhibitor-activated tumor progressive proteins and cell migration *in vitro* and significantly inhibited tumor growth and metastasis *in vivo* [44], suggesting the superior effects of HDAC inhibitor in combination treatment with curcumin.

In LNCaP prostate cancer cells, curcumin demethylated the first 14 CpG sites of CpG island in Neurog1 gene and, in turn, up-regulated the expression of Neurog1 [45].

Curcumin also significantly inhibited MeCP2 (one of the epigenetic modulators) binding to the promoter of Neurog1, leading to decreased expression of Neutog1. Moreover, curcumin inhibited the enrichment of H3K27me3 at the Neurog1 promoter region and the activity of HDAC [45], suggesting the strong effects of curcumin on epigenetic regulation in prostate cancer. In addition, Nrf2 has been found to be a regulator of cellular antioxidant defense system and it is epigenetically silenced during the development of prostate cancer in TRAMP mice. Curcumin could reverse the methylation of the first 5 CpGs in the promoter region of the Nrf2 gene. The demethylation of Nrf2 by curcumin has been found to be correlated with the re-expression of Nrf2 and its target gene NQO-1 [46], suggesting that curcumin could exert its chemopreventive effect through epigenetic modification of the Nrf2-mediated anti-oxidative stress pathway.

In cervical cancer cells, several tumor suppressor genes have been reported to be silenced by promoter methylation. It has been found that curcumin could demethylate the promoter methylation of RARβ2 gene in SiHa cervical cancer cells [47]. In HeLa cervical cancer cells, the hypermethylation of RARβ2 gene was also reversed after 6 days of treatment with curcumin. The reversal of RARβ2-methylation led to the induction of apoptosis. Curcumin could also reverse promoter hypermethylation and increase gene expression of FANCF in SiHa cervical cancer cells. Methylation specific PCR and bisulphite sequencing analysis showed that curcumin was able to demethylate 12 CpG sites in the region of FANCF promoter [48], suggesting the potent demethylating effects of curcumin on tumor suppressor genes.

Wnt inhibitory factor-1 (WIF-1) is another tumor suppressive gene and the hypermethylation of WIF-1 promoter has been found in lung cancer cells and tissues. To reactivate the expression of WIF-1, three major curcuminoids including curcumin, demethoxycurcumin and bisdemethoxycurcumin have been used [49]. It was found that bisdemethoxycurcumin had the strongest demethylation effect *in vitro*. The curcuminoids could restore WIF-1 expression through the demethylation effect [49], suggesting their therapeutic benefit for lung cancer. In acute lymphoblastic leukemia (ALL), it was found that the expression of several genes in the TP53 pathway was decreased due to DNA hypermethylation. The DNA methylation of genes in TP53 pathways was significantly associated with a higher relapse and mortality rate. Importantly, curcumin or Aza-dC treatment reversed the epigenetic abnormalities, resulting in the increased expression of genes in TP53 pathways, and also led to the induction of apoptosis of ALL cells [50], suggesting the epigenetic regulation of tumor suppressors by curcumin.

Histone methyltransferase EZH2 is a critical epigenetic regulator and plays important roles in the control of cell proliferation, apoptosis, and cancer stem cell function. We found that diflourinated-curcumin (CDF), a novel analogue of curcumin, down-regulated the expression of EZH2 and up-regulated the expression of several tumor-suppressive miRNAs including let-7a, b, c, d, miR-26a, miR-101, miR-146a, and miR-200, leading to the inhibition of cell survival, clonogenicity, formation of pancreatospheres, cell migration, and cancer stem cell function in human pancreatic cancer cells [51], suggesting the beneficial effects of CDF on epigenetic regulation.

14.4.3 Epigenetic Regulations by EGCG

EGCG is extracted from green tea and has been shown to have antioxidant and anti-cancer properties. It has been found that EGCG could decrease global DNA methylation in cancer cells. EGCG down-regulated 5-methylcytosine, DNMT1, DNMT3a, and DNMT3b. EGCG also inhibited the activity of histone deacetylase and promoted acetylation in lysine9 and 14 on histone H3 and lysine5, 12 and 16 on histone H4, leading to the up-regulation of silenced tumor suppressor genes, $p16^{INK4a}$ and $p21^{WAF1}$ in A431 cancer cells [52]. EGCG showed its inhibitory effect on the DNMT1-mediated DNA methylation. Computational modeling studies revealed that the gallic acid moiety of EGCG is critical for its inhibitory interaction with the catalytic site of DNMT1.

EGCG could also demethylate the DNA methylation in the promoter regions of several tumor suppressor genes including $p16^{INK4a}$, $p15^{INK4b}$, retinoic acid receptor β (RARβ), O(6)-methylguanine methyltransferase (MGMT), and human mutL homologue 1 (hMLH1) genes, resulting in the up-regulation of these genes in various cancer cells including HT-29 and Caco-2 colon cancer, KYSE 150 esophageal cancer, and PC-3 prostate cancer cells [53]. EGCG could also demethylate the DNA hypermethylation in the promoter region of tumor suppressor WIF-1 gene and restore the expression of WIF-1 in H460 and A549 lung cancer cells [54]. By epigenetic regulation of WIF-1, EGCG decreased the level of cytosolic β-catenin and suppressed the activity of Tcf/Lef reporter, suggesting the inhibitory effects of EGCG on Wnt signaling pathway through the epigenetic mechanism [54]. RECK is also a tumor suppressor gene which down-regulates matrix metalloproteinases (MMPs) and suppresses invasion, angiogenesis and metastasis of cancer. It has been found that EGCG could partially reverse the DNA hypermethylation in the region of RECK promoter and significantly up-regulate the expression of RECK, causing the down-regulation of MMP-2 and MMP-9, and the suppression of invasion in oral squamous cell carcinoma cells [55]. These findings demonstrate the up-regulation of tumor suppressors by EGCG through epigenetic regulation.

It is well known that the status of estrogen receptor-α (ERα) predicts the clinical prognosis and therapeutic outcome in breast cancer. ERα-negative breast cancer commonly has progressive disease and poor prognosis. The silence of ERα is believed to be due to epigenetic regulation in breast cancer cells. It has been found that EGCG could remodel the chromatin structure of the ERα promoter by the inhibition of transcription repressor complex binding to the regulatory region of the ERα promoter [56]. In this way, EGCG has been found to increase the expression of ERα in ERα-negative MDA-MB-231 breast cancer cells. Combination treatment with EGCG and HDAC inhibitor showed a synergistic effect by increasing ERα expression and sensitizing breast cancer cells to tamoxifen, suggesting the beneficial effects of EGCG in the treatment of breast cancer through epigenetic regulation.

It is known that polycomb group (PcG) proteins are epigenetic regulators of gene expression. Multiprotein PcG complexes such as PRC2 and Bmi-1 could up-regulate histone methylation and down-regulate acetylation, resulting in an altered chromatin

conformation and gene expression. In SCC-13 skin cancer cells, the expression and activity of PcG protein were up-regulated with increased cancer cell proliferation and survival. However, the treatment of SCC-13 cells with EGCG significantly inhibited the expression of Bmi-1 and EZH2, leading to reduced cell survival [57]. EGCG treatment could also reduce histone H3 lysine 27 trimethylation through inhibition of PRC2 complex deregulation. The decreased expression of PcG protein by EGCG caused reduced expression of cdk1, cdk2, cdk4, cyclin D1, cyclin E, cyclin A and cyclin B1, and increased expression of $p21^{WAF1}$ and $p27^{kip1}$. Further studies have shown that EGCG could reduce the expression of HDAC1 and the formation of H3K27me3 and H2AK119ub, leading to the up-regulation of tumor suppressors and the suppression of cell survival. The PcG-mediated epigenetic regulation could be one of the molecular mechanisms by which EGCG inhibits skin cancer cell survival.

EGCG could also regulate acetylation of NF-κB. It is known that p300/CBP-mediated hyperacetylation of RelA (p65) promotes the activation of NF-κB in cancer cells. EGCG could inhibit the acetylation of p65 and abrogate p300-induced p65 acetylation *in vitro* and *in vivo*, leading to the inhibition of NF-κB activation [58]. By the inhibition of p65 hyperacetylation, EGCG suppressed TNFα-induced p65 nuclear translocation. Furthermore, EGCG decreased the p300 binding to IL-6 promoter with an increased recruitment of HDAC3 [58]. These results demonstrate that EGCG could regulate NF-κB signaling by epigenetic regulation.

EGCG has also been found to down-regulate telomerase activity in breast cancer cells through the inhibition of hTERT by epigenetic mechanisms. EGCG decreased the level of acetyl-H3, acetyl-H3K9, and acetyl-H4 in the hTERT promoter and modulated chromatin structures of the hTERT promoter [59]. Moreover, EGCG promoted hTERT repressors including MAD1 and E2F-1 binding to the hTERT regulatory region. Furthermore, EGCG could demethylate DNA hypermethylation in the promoter of CTCF and increase the expression of CTCF which down-regulates hTERT expression by binding to hTERT promoter. These findings all suggest the effects of EGCG on epigenetic regulation in multiple cancers.

14.4.4 Epigenetic Regulations by Resveratrol

Resveratrol is a dietary compound from grapes and shows anti-carcinogenic activity. It has been found that resveratrol could epigenetically regulate the expression of several tumor suppressor genes. The BRCA1 protein is a tumor suppressor, especially in breast cancers. Aromatic hydrocarbon receptor (AhR) could down-regulate the expression of BRCA1. The activation and recruitment of AhR to BRCA1 promoter blocked the expression of BRCA1 with reduced acetylated histone 4 and AcH3K9, and increased DNMT1 and MBD2. However, this AhR-dependent repression of BRCA1 expression could be reversed by resveratrol treatment [60], suggesting that epigenetic silencing of BRCA1 gene could be prevented by resveratrol. Moreover, resveratrol could inhibit the function of tumor promoter, 2,3,7,8

tetrachlorodibenzo-p-dioxin (TCDD). It has been found that TCDD could inhibit 17β-estradiol-dependent stimulation of BRCA1, and could also induce hypermethylation of CpG sites that has been found in the start site of BRCA1 transcription, leading to the lower expression of BRCA1 in breast cancer cells. Therefore, resveratrol treatment could epigenetically reactivate BRCA1 by inhibition of AhR/TCDD/DNMT1 signaling [61]. In addition, it has been found that BRCA1 binds to the SIRT1 promoter and promotes the expression of SIRT1, which in turn suppresses survivin by epigenetic modification of histone H3. Resveratrol could increase the expression of Sirt1 and, in turn, could down-regulate the expression of survivin, suggesting that resveratrol treatment combined with conventional chemotherapeutics could be a strategy for the treatment of BRCA1-negative breast cancer [62]. Moreover, Resveratrol could inhibit RASSF-1α DNA methylation and, in turn, increase the expression of RASSF-1α, leading to the inhibition of prostaglandin PGE_2 in breast cancers [63], suggesting the beneficial effects of resveratrol in the epigenetic regulation of tumor suppressors in breast cancer.

Resveratrol could also inhibit the expression of some oncogenes which participate in epigenetic regulations. Metastasis-associated protein 1 (MTA1) is an oncogenic protein which promotes deacetylation of histones. It has been shown that MTA1 is overexpressed in prostate cancer and its overexpression is associated with tumor aggressiveness and metastasis. It has been found that resveratrol could decrease the expression of MTA1, leading to the acetylation and activation of p53 [64]. The acetylated p53 could recruit to $p21^{WAF1}$ and Bax promoters, resulting in the apoptosis of cancer cells. HDAC inhibitor SAHA shows similar effects as resveratrol, suggesting the epigenetic regulation of resveratrol in cancer cells [64]. It has also been found that lysine acetylation of the oncogenic transcription factor STAT3 is increased, leading to the high expression of STAT3 in cancers. Resveratrol could reduce acetylation of STAT3 at Lys685 and, in turn, increase the expression of several tumor-suppressor genes, leading to the inhibition of cancer growth. The reduction of acetylated STAT3 also caused demethylation and activation of ERα, which could sensitize triple-negative breast cancer cells to anti-estrogen therapy [65].

In addition, it has been shown that viruses, including HIV-1, could increase the expression of human DNA methyltransferases, leading to the development of cancers. Interestingly, the HIV-1 induced overexpression of DNA methyltransferase could be prevented with resveratrol treatment through the inhibition of transcription factor AP1 signaling [66], suggesting the chemopreventive effects of resveratrol through epigenetic regulation.

14.4.5 Epigenetic Regulations by I3C and DIM

I3C and its *in vivo* dimeric product DIM are phytochemicals derived from cruciferous vegetables and has been shown to have no known toxicity in humans. Both I3C and DIM could inhibit carcinogenesis in different types of cancers. In recent years,

HDAC inhibitors have been synthesized for cancer prevention and therapy; however, the side effects and toxicity limits the use of HDAC inhibitors in humans. Interestingly, it was found that both I3C and DIM could inhibit HDAC activity in prostate cancer cells [67]. I3C modestly inhibited HDAC activity in androgen sensitive LNCaP cells whereas DIM significantly inhibited the expression of HDAC2 and reduced the activity of HDAC with increased expression of $p21^{WAF1}$ in both LNCaP and PC-3 cells, suggesting that DIM is a better natural agent for the regulation of aberrant epigenetic patterns in prostate cancer prevention or treatment [67]. We have also found that DIM treatment could demethylate the DNA methylation in the promoter of miR-34a, leading to the up-regulation of miR-34a expression and the down-regulation of target genes, AR (downstream targets of AR, PSA) and Notch 1 in LNCaP and C4-2B cells [15]. Moreover, DIM intervention in prostate cancer patients prior to radical prostatectomy resulted in the re-expression of miR-34a and consequently led to decreased expression of AR, PSA and Notch-1 in prostate tumor tissues [15]. These results suggest that epigenetic silencing of tumor suppressive miR-34a in prostate cancer could be reversed by DIM treatment.

The overexpression of oncogenic cyclooxygenase-2 (COX-2) has been found in several types of cancers with activation of AhR signaling. It was found that AhR ligand could induce the rapid formation of complex with the AhR, the histone acetyl transferase p300, and acetylated histone H4 at the COX-2 promoter [68]. Importantly, DIM could inhibit the recruitment of AhR and acetylated histone H4 to the COX-2 promoter and, thereby, down-regulate the expression of COX-2 in MCF-7 breast cancer cells, suggesting that the use of DIM could be a novel strategy against epigenetic activation of COX-2 by AhR.

14.4.6 Epigenetic Regulations by Lycopene

Lycopene is the red pigment in tomatoes and has shown its chemopreventive potential in cancer research. The effects of lycopene on DNA methylation in the promoter of tumor suppressor genes have been tested in MDA-MB-468 breast cancer cells and MCF10A breast epithelial cells. It was found that lycopene partially demethylated the DNA hypermethylation in the promoter of glutathione S-transferase P1 (GSTP1) tumor suppressor gene in MDA-MB-468 cells. The expression of GSTP1 was significantly up-regulated after lycopene treatment. However, the demethylation of another tumor suppressor gene RARβ by lycopene was only observed in noncancerous MCF10A breast epithelial cells [69]. A controversial observation has been reported in prostate cancer cells. GSTP1 has been found to be hypermethylated in 90 % of prostate cancers; however, lycopene was unable to alter the methylation and expression of GSTP1 in LNCaP prostate cancer cells while a demethylating agent was able to significantly decrease the methylation of GSTP1 gene [70]. These results suggest that the effects of lycopene on epigenetic regulation could be cell type and context-dependent.

14.5 Conclusions and Perspectives

Mounting evidence suggests that epigenetic regulations of mRNAs and miRNAs by DNA methylation and histone modification play important roles in cancer development and progression; therefore, targeting the epigenetic deregulations in cancers is a key and effective approach to fight against cancers. Several epigenetic drugs or HDAC inhibitors have been synthesized and used in epigenetic therapy trials to re-express abnormally silenced tumor suppressor genes. However, the adverse side-effects of the demethylating agents and histone deacetylase inhibitors limit the broader application of these agents to appreciate their beneficial effects. Therefore, using non-toxic nutraceuticals including isoflavone, curcumin, EGCG, resveratrol, I3C, DIM, and lycopene to demethylate DNA sequences or inhibit HDACs could epigenetically deregulate the expression of tumor suppressive mRNAs and miRNAs.

Indeed, the *in vitro* experiments and *in vivo* animal studies have demonstrated that the epigenetic regulation of mRNAs and miRNAs could be one of the molecular mechanisms by which nutraceuticals could inhibit carcinogenesis and cancer progression. These natural agents exert their potent effects on the inhibition of cancer cell growth, invasion, and metastasis partly mediated through epigenetic regulation, suggesting that these non-toxic agents having anti-cancer effects could be useful in combination treatment with conventional chemotherapeutics for the treatment of cancers. It is important to note that recent development of technologies such as next-generation of sequencing coupled with chromatin immunoprecipitation (ChIP-seq) and DNA methylation profiling will lead to a deeper understanding of the epigenetic regulations in cancers and the effects of nutraceuticals on epigenome. However, more mechanistic experiments and clinical trials are needed to appreciate the value of nutraceuticals in cancer prevention and treatment which is mediated in part due to their roles in epigenetic deregulation of genes relevant to human cancers.

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Chapter 15
Towards Curative Cancer Therapy with Maspin: A Unique Window of Opportunity to Target Cancer Dormancy

Alexander Kaplun, M. Margarida Bernardo, Sijana Dzinic, Xiaohua Li, Ivory Dean, Benjamin Jakupovic, Jason Liu, Elisabeth Heath, Wael Sakr, and Shijie Sheng

Abstract Tumor dormancy is considered to be the last frontier in the battle to cure cancer. Although experimental evidence and clinical studies led to some consensus regarding the phenotypical characteristics of tumor dormancy, the underlying biological controls remain elusive. As a result, in the absence of dormancy-targeted therapeutic strategies, cancer drug resistance and recurrence are a certainty in a matter of time. In this review, we discuss a novel opportunity to target prostate tumor dormancy based on the expression of tumor suppressor maspin, an epithelial-specific endogenous inhibitor of histone deacetylase 1 (HDAC1).

Keywords Prostate cancer • Epigenetics • Gene expression profiling • Bioinformatics • Metastasis • Tumor cell plasticity • Experimental scheme of tumor dormancy • Therapeutic development

Tumor dormancy is considered to be a protracted quiescent state wherein the presence of tumor cells is not linked to disease appearance [1]. Having acquired the capacity to metastasize, dormant tumor cells derived from metastatic sites are arguably the

A. Kaplun • M. Margarida Bernardo • S. Dzinic • X. Li • I. Dean
B. Jakupovic • J. Liu • W. Sakr
Department of Pathology, Wayne State University School of Medicine and Karmanos Cancer Institute, 4100 John R St, Detroit, MI 48201, USA

E. Heath
Department of Oncology, Wayne State University School of Medicine and Karmanos Cancer Institute, 4100 John R St, Detroit, MI 48201, USA

S. Sheng (✉)
Department of Pathology, Wayne State University School of Medicine and Karmanos Cancer Institute, 4100 John R St, Detroit, MI 48201, USA

Department of Oncology, Wayne State University School of Medicine and Karmanos Cancer Institute, 4100 John R St, Detroit, MI 48201, USA
e-mail: ssheng@med.wayne.edu

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last frontier of cancer therapy. Unfortunately, the current therapies that target DNA replication, angiogenesis or host immune response are not effective in eradicating dormant tumor cells, leaving them the time to eventually initiate a metastatic recurrence. Here we will summarize and reflect on the current consensus of the origin of tumor dormancy, with a focus on tumor epigenetics. Based on the accumulated evidence regarding tumor suppressor gene maspin, we raise a novel hypothetical model in which dormant tumor cells that express maspin and consequently exhibit "better-differentiated" phenotype can be targeted based on maspin-controlled epigenetics in the metastatic microenvironment.

15.1 Tumor Dormancy-Focus on Epigenetics

Compared to indolent tumor cells that do not progress to malignancy, the quiescent phenotype of dormant tumor cells is deceptive. According to the current literature, the mechanisms of how metastatic tumor cells revert into a better-differentiated and quiescent phenotype involve a lack of angiogenesis and sensitivity to immune surveillance [1]. However, these phenotype-based concepts of tumor dormancy may have confused the chicken with the egg. It is worth noting that regardless of how the tumor cells interact with the tumor microenvironment, the dormant tumor cells have already gone through a continuum of tumor metastasis. Therefore they are endowed with the capacity to stimulate angiogenesis, and engage immune cells that cause local inflammatory extracellular remodeling. It is also important to note that when dormant tumor cells become reactivated, the recurrent tumor cells will initiate angiogenesis and evade immune surveillance. Thus, the criteria used to define tumor dormancy are actually merely consequences, rather than the causes.

Dormant cells may derive from induced mesenchymal-to-epithelial transition (MET) of tumor cells at the site of metastasis, or directly derive from a small population of cancer stem cells. Although cancer genetics may be the dominant driving force of oncogenesis and metastatic potential, it is important to keep in mind that genetic changes are irreversible and cannot explain the full spectrum of plastic tumor behavior associated with the epithelial-to-mesenchymal transition (EMT), metastasis and MET involved in tumor dormancy. To this end, the definition of tumor dormancy by histological or cellular characteristics does not help devise specific treatments to target dormancy, since dormant tumor characteristics bear significant similarities with indolent tumor cells or normal epithelial cells.

15.2 Maspin-Controlled Epigenetics and Tumor Dormancy

Permanent changes, such as oncogene amplification or tumor suppressor gene deletion cannot be reversed and therefore, cannot be the immediate reasons for the phenotypical plasticity involved in tumor dormancy. Tumor dormancy is likely to be

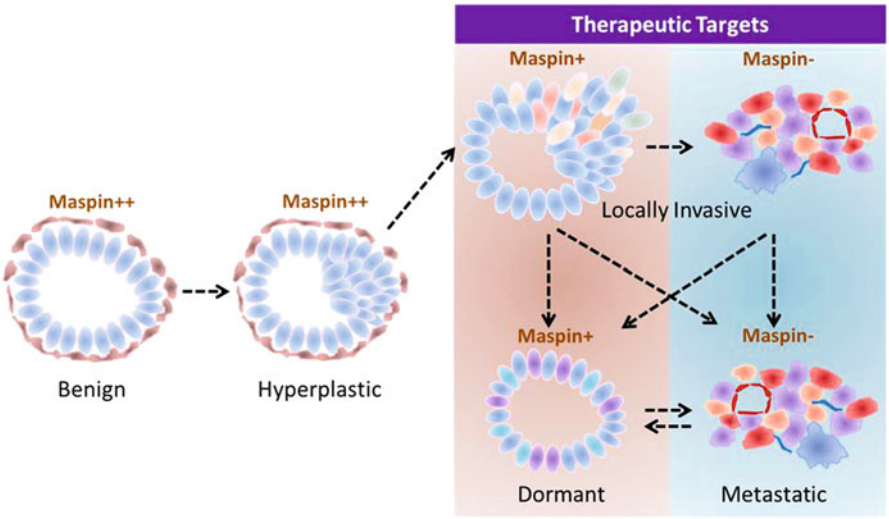

Fig. 15.1 A hypothetical model for how maspin may be used to stratify tumor cells in different differentiation states in tumor progression

driven by changes of epigenetics and microenvironment-dependent. The identification of a gene expression signature specifically associated with tumor dormancy will be invaluable in the development of tumor dormancy-targeted therapy. Dormant tumor cells bear a similar gene expression signature as differentiated epithelial cells, including gap junction proteins and E-cadherin [2, 3]. For this shared similarity, it would be impractical to use the re-expression of these genes as a way of specific detection or targeting dormant tumor cells. Ideally, we need to rely on a gene expression signature that is not only consistent with the "better-differentiated phenotype", but also reflects the pathological context that distinguishes dormant tumor cells from the normal cells or indolent tumor cells.

We have been investigating the clinical relevance and biological function of tumor suppressor maspin, an epithelial-specific endogenous inhibitor of histone deacetylase 1 (HDAC1). As summarized in Fig. 15.1, maspin is epigenetically down-regulated in tumor progression [4, 5]. In experimental tumor models, maspin has been shown to inhibit tumor growth, restore epithelial differentiation, block invasion and metastasis *in vivo*, and sensitize tumor cells to drug-induced apoptosis [6–10]. Interestingly, in human breast cancer, maspin was also found re-expressed in dormant cancer cells with better differentiated phenotype [3].

To understand the contextual association of maspin in dormancy, it is critical to develop a new strategy to study the effects of maspin in tumor dormancy. The key experimental system we used takes advantage of human prostate cancer cell line DU145 that does not express maspin. Maspin transfected DU145 cells *vs*. the mock transfected control, displayed a progressively more differentiated epithelial phenotype when the cells were cultured in 3-dimensional (3D) collagen I matrix and in xenograft tumors using the Scid-Hu model for human prostate cancer bone

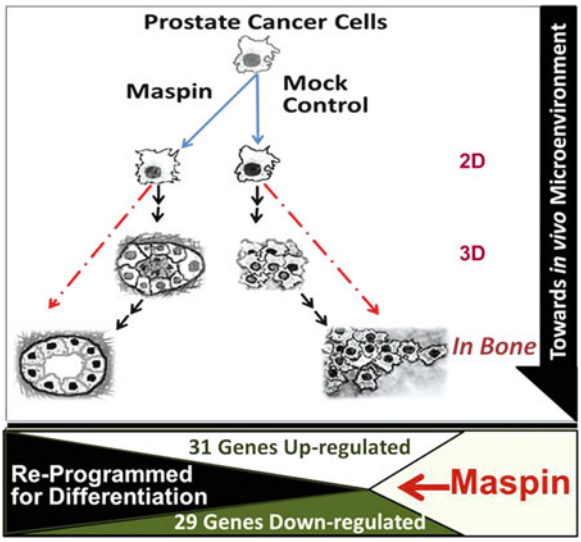

Fig. 15.2 An experimental model for identifying epigenetic networks that is unique for tumor dormancy

metastasis (*in vivo* bone tumor) compared to monolayer culture (2D) where no differentiation occurred [11]. These data support a role of maspin in controlling epithelial homeostasis, especially when tumor cells are grown in environments that closely resemble the *in vivo* microenvironment. Microarray profiles of gene expression were obtained to investigate these possibilities [11].

As summarized in Fig. 15.2, genome-wide RNA microarray of the prostate cancer cell line DU145 transfected with maspin or mock plasmid, identified 31 commonly up-regulated genes and 29 commonly down-regulated genes by maspin, in all three experimental systems (2D, 3D and *in vivo* xenograft bone tumor) [11]. These core changes are likely to be the central coordinators involved in epithelial differentiation or tumor cell redifferentiation program. Overall, the maspin-induced gene expression profile supports the role of maspin as an endogenous HDAC1 inhibitor since 18 out of 31 genes are HDAC1- target genes. Moreover, our present data is consistent with our earlier findings that maspin expression increased the expression of p21, cytokeratin 8 and 18 (all 3 HDAC1 target genes) and decreased the expression of VEGF, uPA, and uPAR [11–13].

Based on the biochemical properties of maspin, the core set of the genes responsive to maspin may be expressed as long as maspin is present. Thus, maspin may tightly control epithelial homeostasis. This set of genes is likely present in all maspin-expressing cells including normal epithelial cells, primary tumor cells with better differentiated phenotypes, and metastatic tumor cells that are reverted back to better-differentiated phenotypes (dormancy) in secondary organ/tissue microenvironments For this reason, this set of genes will not be useful for distinguishing between indolent diseases from dormancy.

Bioinformatics analysis also identified additional maspin-induced changes in gene expression specifically associated with the tumor phenotypical changes in 3D

Table 15.1 Specific changes induced by maspin in prostate tumor cells in bone

	No of genes	Transcriptional factors
UP-regulated	81	HNF1(A,B), Fox, CDX(1.2), E2F6, HOXA1, Nanog, Hmga1, Onecut(1,2), Irf(1,2) and Mds1
Down-regulated	92	Nanog, CDX(1,2), Fox, ARID5B, Mef2(a-d), ZFHX3, Prrx2, Pax4, TFAP4, MXX1, Pou2f1, Cebpg

collagen culture and bone microenvironment, respectively [11]. As summarized in Table 15.1, the unique epigenetic changes induced by maspin in bone microenvironment include the up-regulation of 81 genes and down-regulation of 92 genes. These genes are mostly associated with stress-response, cell differentiation, and repression of tumor-induced stromal reactivities. While these profiles reveal the molecular events and context reprogrammed by maspin towards epithelial differentiation, they do not automatically specify the pathways that are controlled by maspin. To get more insights into the molecular mechanisms underlying the epigenetic effect of maspin, it is important to determine what factors mediated the maspin effects on gene transcription. We employed a comprehensive bioinformatic approach utilizing the gene regulation analysis tools TRANSFAC [14] and ExPlain [15] developed by Biobase. The results of our search are listed in Table 15.1. It is likely that the combination of maspin and additional specific pathological insults at the site of bone metastasis can activate a different set of transcription factors, and release a different set of HDAC target genes from repression, providing added benefit for tumor cell survival. In the absence of maspin, cells may not be equipped with this flexibility of epigenetic reprogramming and will not reverse from the metastatic phenotype to a dormant quiescent phenotype. Currently, we are further validating the expression of these genes and transcription factors in human specimens, and investigating the possible concerted biological functions of these genes in dormant *vs.* metastatic tumor phenotypes.

At the center of tumor dormancy is cellular plasticity which is also linked to the concept of tumor cell stemness. This notion is supported by some evidence. For example, stem cell markers Oct4, CD44, and CD24 have been shown to be associated with breast cancer reversion to dormancy [16]. Our finding that maspin expression regulates the expression of another stem cell regulator, nanog, suggests that maspin is directly involved in the re-orientation of tumor cells to a commitment to differentiation.

Judging from the phenotypical characteristics and molecular markers that have been implicated in dormancy, it is conceivable that dormant tumor cells are partially differentiated by expressing some tumor suppressors such as maspin and gap junction proteins. This notion may cause conceptual and practical confusion about what is good and what is bad in tumor progression. From a practical point of view, the better prognosis associated with the detection of epithelial differentiation markers does not exclude dormancy-related remission. Thus, while those good prognostic markers may have clinical significance, it is the context of these markers in dormant tumors that confer the added potential of drug resistance and tumor recurrence.

It is important to note that maspin is the only endogenous polypeptide HDAC1 inhibitor identified thus far. HDAC1 is the most abundant class I HDAC in mammalian cells, which is up-regulated in many types of cancer including prostate cancer [17]. Our evidence demonstrates that maspin differs significantly from pharmacological HDAC inhibitors at least in three important aspects. First, genes that are commonly regulated by maspin are significantly fewer than those affected by pharmacological inhibitors. Second, HDAC target genes commonly regulated by maspin are clustered to function in epithelial differentiation. Lastly, the endogenous regulation of HDAC target genes by maspin is sensitive to changes of tissue microenvironments, and may be used as specific markers and/or targets for tumor cells that survive in metastatic sites.

It remains to be clearly dissected how genomic structures composed of the same histone proteins that have the same potential to be regulated at the biochemical level by histone-modifying enzymes could be differentially regulated by the endogenous HDAC inhibitor maspin. The genomic hierarchical structure and the composition of the HDAC complex may be the key in determining the specificity of transcriptional activity, depending on the topological presentation of histones and the relative abundance and composition of the transcription complex. This general order of hierarchy of site-specific DNA activity and regulation is not unique for cancer cell plasticity. In fact, a higher-order of DNA structure is required and essential to determine the flow of information and sequence of phenotypical changes in development [18]. For example, HDAC1 was shown to only colocalize with specific sites of chromosomes during meiosis, and plays a critical role of propagating the gene expression patterns to the descendent generation [19].

15.3 Future Perspectives of Maspin-Based Anti-tumor Dormancy Therapies

It is reasonable to believe that tumor drug sensitivity may not be predominantly determined by tumor cell genetics. Rather, it could be a function of the epigenetic dynamics that varies at different stages of tumor progression. The evidence that a specific set of genes and the corresponding up-stream transcription factors have been linked to maspin only in the bone microenvironment raises an exciting possibility that these genes and factors may be targeted for eradicating dormant tumor cells. We can argue further, in general, that the epigenetics that supports a better-differentiated phenotype may be more drug-resistant, as in the case of tumor dormancy [20]. As illustrated in Fig. 15.3, we propose that epigenetics that underlies the phenotypical characteristics and metastatic potential may be stratified based on the expression of maspin and maspin-associated genes. Maspin is epithelial-specific gene. Epithelial cells that express maspin may include normal epithelial cells, indolent tumor cells, as well as dormant tumor cells. Maspin is down-regulated in invasive and metastatic tumor cells [21]. In addition, non-epithelial cell types can be recognized for lack of maspin expression.

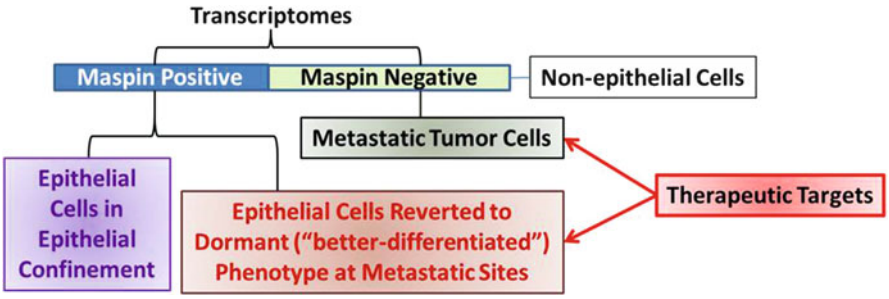

Fig. 15.3 A strategy towards curative cancer treatment by targeting maspin-negative metastatic tumor cells and maspin-positive dormant tumor cells

While maspin alone may not be a definitive lineage-tracker, the maspin-associated transcriptomes may stratify dormant tumor cells from other maspin-expressing cells, as suggested by our experimental evidence. In tumor progression, as compared to those tumor cells that do not express maspin and do not revert to a dormant phenotype, re-expression of maspin in dormant tumor cells may lead to distinct transcriptome to be targeted by cancer therapy. Interestingly, we have shown with cells in monolayer culture that maspin expression specifically increased tumor, but not normal, cell sensitivity to the cytotoxicity of chemotherapeutic agents. Normal cells that expressed a high level of maspin seemed more drug-resistant. It will be important to test whether the re-differentiation of tumor cells induced by maspin renders the tumor cells more resistant to conventional chemotherapies at the site of metastasis. If that is the case, a focus on maspin-associated novel and unique drug targets may open a new window of opportunity to target tumor cells in a dormant state.

Our strategy to stratify tumor cells based on maspin and maspin-associated transcriptome is aligned with potential application for specific detection and therapeutic treatment of dormant tumor cells. In combination with the conventional and molecular-targeted therapies that target proliferation, tumor inflammation, and metastatic phenotypes, therapeutic strategies that target tumor dormancy may finally eradicate both metastatic and dormant tumor cells by blocking tumor progression and preventing tumor recurrence simultaneously. Our specific conclusion based on these studies need to be further validated, and may not be readily applicable to dormancy of prostate tumor at other metastatic sites compared to bone or dormancy of other types of tumor. However, considering the difficulties to acquire and conduct functional/biological studies with match-paired human specimens from primary tumor, metastatic tumor and dormant tumor, our current experimental scheme may serve as a model for how dormancy-specific epigenetics can be identified and tested.

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