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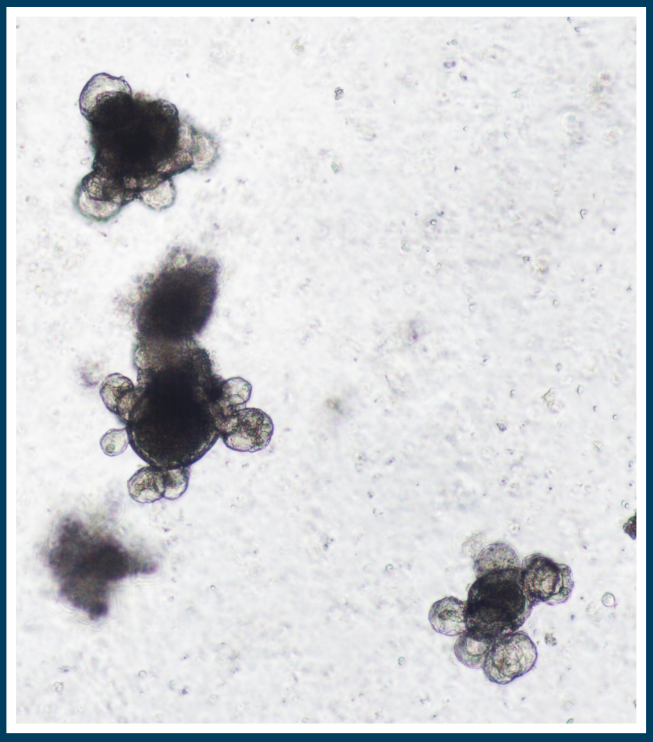

ALTERATIONS OF EPIGENETICS AND MICRORNAS IN CANCER AND CANCER STEM CELL

Topic Editor
Yoshimasa Saito

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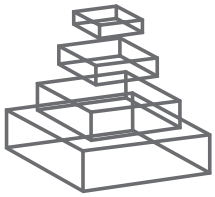

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ALTERATIONS OF EPIGENETICS AND MICRORNAS IN CANCER AND CANCER STEM CELL

Topic Editor:
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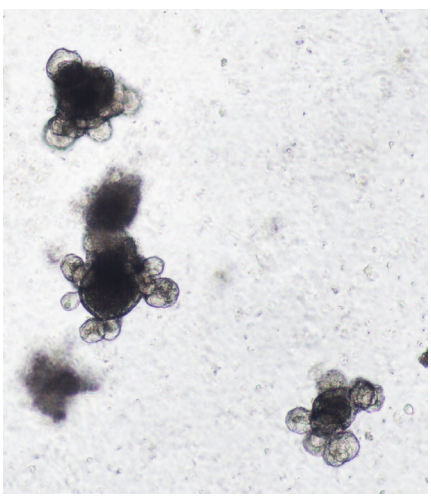

Organoid culture of stem cells derived from mouse intestine

Studies have shown that alterations of epigenetics and microRNAs (miRNAs) play critical roles in the initiation and progression of human cancer. Epigenetic silencing of tumor suppressor genes in cancer cells is generally mediated by DNA hypermethylation of CpG island promoter and histone modification such as methylation of histone H3 lysine 9 (H3K9) and tri-methylation of H3K27. MiRNAs are small non-coding RNAs that regulate expression of various target genes. Specific miRNAs are aberrantly expressed and play roles as tumor suppressors or oncogenes during carcinogenesis. Important tumor suppressor miRNAs are silenced by epigenetic alterations, resulting in activation of target oncogenes in human malignancies.

Stem cells have the ability to perpetuate themselves through self-renewal and to generate mature cells of various tissues through differentiation. Accumulating evidence suggests that a subpopulation of cancer cells with distinct stem-like properties is responsible for tumor initiation, invasive growth, and metastasis formation, which is defined as cancer stem cells. Cancer stem cells are considered to be resistant to conventional chemotherapy and radiation therapy, suggesting that these cells are important targets of cancer therapy. DNA methylation, histone modification and miRNAs may be deeply involved in stem-like properties in cancer cells. Restoring the expression of tumor suppressor genes and miRNAs by chromatin modifying drugs may be a promising therapeutic approach for cancer stem cells. In this Research Topic, we discuss about alterations of epigenetics and miRNAs in cancer and cancer stem cell and understand the molecular mechanism underlying the formation of cancer stem cell, which may provide a novel insight for treatment of refractory cancer.

Table of Contents

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EDITORIAL
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Alterations of epigenetics and microRNAs in cancer and cancer stem cell

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Keywords: epigenetics, microRNAs, cancer, cancer stem cells, methylation

ALTERATIONS OF DNA METHYLATION AND HISTONE MODIFICATION IN CANCER

Epigenetics is an acquired modification of methylation and/or acetylation of chromatin DNA or histone proteins, which regulates downstream gene expression. Epigenetic alterations can be induced by aging, chronic inflammation and viral infection. Aberrant DNA methylation and/or histone modification at the CpG island promoter may induce inactivation of tumor suppressor genes and play critical roles in the initiation and progression of human cancer. *In silico* analysis is essential to investigate putative genetic and epigenetic elements of tumor suppressor genes such as *Rb1* gene. This may contribute genetic and epigenetic information modulating tissue-specific transcripts and expression levels of genes (Hajjari et al., 2014). Genome-wide analysis of DNA methylation by BeadChip assay is quite useful to identify aberrantly methylated genes in human cancers. *HIST1H3J*, *POU4F2*, *SHOX2*, *PHKG2*, *TLX3*, and *HOXA7* were identified as aberrantly methylated genes in human papillary thyroid cancers by genome-wide analysis of DNA methylation. In addition, papillary thyroid cancers with preferential methylation were significantly associated with mutations of the BRAF/RAS oncogenes. These hypermethylated genes may constitute potential biomarkers for papillary thyroid cancer (Kikuchi et al., 2013).

In 2010, the International Human Epigenome Consortium (IHEC) was established to coordinate the production of reference maps of human epigenomes for key cellular states (http://www.ihec-epigenomes.net/). In order to gain substantial coverage of the human epigenome, the IHEC is planning to decipher at least 1000 epigenomes. These multilayer-omics analyses including genome, epigenome, transcriptome, proteome and metabolome are important for elucidating the molecular carcinogenesis and for exploring biomarkers and therapeutic targets for human cancers (Kanai and Arai, 2014).

DYSREGULATION OF microRNAs (miRNAs) BY EPIGENETIC ALTERATIONS IN CANCER

miRNAs are a class of endogenous non-coding RNAs that play an important role in the regulation of several cellular, physiological and developmental processes. Aberrant miRNA expression is associated with many human diseases including cancer. Specific miRNAs are aberrantly expressed and play roles as tumor suppressors or oncogenes during carcinogenesis. Barrett's esophagus is considered to be a complication of gastroesophageal reflux disease and a precursor lesion of esophageal adenocarcinoma. Expression levels of *miR-221* and *miR-222* were increased when cultured esophageal epithelial cells were exposed to bile acids, which is one of the risk factors of esophageal adenocarcinoma. These miRNAs are known to specifically target p27Kip1, which inhibits the degradation of CDX2. Thus the degradation of CDX2 was enhanced by up-regulation of *miR-221* and *miR-222* on exposure of esophageal epithelial cells to bile acids (Matsuzaki and Suzuki, 2014).

Important tumor suppressor miRNAs are silenced by epigenetic alterations, resulting in activation of target oncogenes in human malignancies. But some oncogenic miRNAs such as *miR-196* family, *miR-200* family and *miR-519d* are reported to be up-regulated via DNA hypomethylation in various cancers. Histone modifications also play important roles in the dysregulation of miRNAs. Conversely, dysregulation of miRNAs such as *miR-152*, *miR-29* family and *miR-101* is related to epigenetic alterations through targeting chromatin-modifying factors including DNMT1, DNMT3A, DNMT3B, and EZH2 in cancer. Aberrant methylation of miRNA genes could be a potential biomarker for detecting cancer and predicting its outcome (Suzuki et al., 2013). Several miRNAs are dysregulated in lung cancers in response to DNA methylation and histone modification including methylation of histone H3 lysine 9 (H3K9) and H3K27. In lung cancer, several miRNAs such as *miR-9* and *miR-34* family are silenced by DNA methylation, whereas *miR-212* is silenced by methylation of H3K9 and H3K27 rather than DNA methylation (Watanabe and Takai, 2013).

ALTERATIONS OF EPIGENETICS AND miRNAs IN CANCER STEM CELL

Stem cells have an ability to perpetuate themselves through self-renewal and to generate mature cells of various tissues through differentiation. Accumulating evidence suggests that a subpopulation of cancer cells with distinct stem-like properties is responsible for tumor initiation, invasive growth, and metastasis formation, which is defined as cancer stem cells (CSCs). CSCs express specific cell surface markers including CD44, CD133, and EpCAM. Recently, a novel 3D culture method for stem cells called "organoid culture" has been developed. This culture method uses a serum-free medium that includes only identified growth factors such as R-spondin 1, EGF, and Noggin. R-spondin 1 is a ligand for Lgr5, which is a marker for intestinal stem cells and an essential factor to activate Wnt signal in intestinal crypts. Intestinal organoid culture enabled to expand normal or tumor epithelial

cells *in vitro* with stem cell properties. This model will become a powerful research tool in clarifying the molecular pathogenesis and drug susceptibility of CSCs. Manipulation of cancer-related genes in stem cells may reveal the molecular mechanism underlying human carcinogenesis (Fujii and Sato, 2014). On the other hand, the role of mesenchymal stem cells (MSCs) in cancer development is still controversial. MSCs may promote tumor progression through immune modulation, but other tumor suppressive effects of MSCs have also been reported. Since systemically administered MSCs can be recruited and migrated toward tumors, the incorporation of engineered MSCs can be used as novel anti-tumor carriers for the development of tumor-targeted therapies (Yagi and Kitagawa, 2013).

miRNAs including *let-7* and *miR-34a* have been implicated in the regulation of CSC properties by suppression of their target genes such as HMGA2, RAS, NOTCH1, and CD44. The modulation of CSC gene expression by miRNAs could be a novel therapeutic strategy targeting CSCs (Takahashi et al., 2014). Glioblastomas show heterogeneous histological features, which are considered to be associated with the presence of glioma stem cells (GSCs). GSCs have an ability to self-renew and initiate the growth of gliomas and are resistant to conventional chemotherapies. The oncogenic miRNAs including *miR-17-92* cluster is involved in the regulation of GSC differentiation, apoptosis and proliferation by suppression of target genes such as CTGF. The tumor suppressor miRNAs including *miR-34a* is also dysregulated in GSCs. *miR-34a* directly inhibits the expression of c-Met, Notch-1 and Notch-2 and involved in the differentiation of GSCs. Long non-coding RNAs (lncRNAs) such as *MEG3* and *CRNDE* are also dysregulated in glioma tissues and may be associated with the stemness of glioma cells (Katsushima and Kondo, 2014).

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Culturing intestinal stem cells: applications for colorectal cancer research

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Recent advance of sequencing technology has revealed genetic alterations in colorectal cancer (CRC). The biological function of recurrently mutated genes has been intensively investigated through mouse genetic models and CRC cell lines. Although these experimental models may not fully reflect biological traits of human intestinal epithelium, they provided insights into the understanding of intestinal stem cell self-renewal, leading to the development of novel human intestinal organoid culture system. Intestinal organoid culture enabled to expand normal or tumor epithelial cells *in vitro* retaining their stem cell self-renewal and multiple differentiation. Gene manipulation of these cultured cells may provide an attractive tool for investigating genetic events involved in colorectal carcinogenesis.

Keywords: cancer stem cells (CSC), wnt proteins, R-spondin, organoids, niche

INTRODUCTION

Despite recent advances in therapeutics, colorectal cancer (CRC) is a major health issue; more than a million people develop CRC, causing more than 700 thousand deaths worldwide yearly (Lozano et al., 2012). Surgically non-resectable tumors or metastatic disease ultimately acquires resistance to therapy, leading to death (Cunningham et al., 2010). The notion that a limited number of cells within a cancer are exclusively capable of initiating and maintaining the tumor, i.e., the cancer stem cell (CSC) hypothesis, has recently been gaining favor, and CRC is no exception. CSCs are referred to as being resistant to therapy, responsible for tumor metastasis and recurrence, and potential targets of new therapeutic strategies. Investigators have attempted to identify or isolate colorectal CSCs; however, direct evidence of colorectal CSCs has been lacking to date (Clevers, 2011).

Recently, crypt base columnar cells (CBC cells) lying at the bottom of intestinal crypts were shown to give rise to all lineages of intestinal epithelial cells by genetic tracing of the *Lgr5* gene (Barker et al., 2007). Genetic transformation of these Lgr5$^+$ intestinal stem cells (ISCs) has shown their potential as tumor-initiating cells (Barker et al., 2009). A method of maintaining and expanding ISCs *ex vivo* has also been established (Sato et al., 2009). This dramatic progress has provided new insight into ISC biology and may prove useful in understanding the relationship between ISCs and colorectal CSCs.

IDENTIFICATION OF INTESTINAL EPITHELIUM STEM CELLS

The intestinal epithelium is one of the most rapidly renewing tissues in the adult mammalian body, with complete turnover every 4–5 days (Barker et al., 2008). The small intestine epithelium comprises two histologically distinct structures: the villi projected toward the gut lumen, and the crypts invaginating into the mucosa. The villus contains three types of post-mitotic differentiated intestinal cells with the divergent functions of absorption (enterocytes), mucus secretion (goblet cells), and hormone secretion (endocrine cells). Paneth cells, which secrete lysozyme, reside at the base of the crypt. The colorectal epithelium lacks villi and Paneth cells, although the general structure remains similar to that of the small intestine.

The existence of long-lived ISCs capable of generating all other types of intestinal cells was first proposed by Stevens and Leblond (1947). Their pulse-chase analysis of ^{3}H thymidine-labeled proliferating cells by autoradiography demonstrated that continuously proliferating intestinal crypt cells completely replace the villus cells every 3 days. This finding later led to the concept that all differentiated intestinal cell types ultimately originate from undifferentiated cells residing at the bottom of the crypt, specifically, the crypt base columnar cells interspersed between the Paneth cells (Cheng and Leblond, 1974).

Subsequent work by Potten et al. (1978) found that CBCs residing at position +4 relative to the crypt bottom retained the radio-isotopic DNA label, suggesting that these cells were very slowly dividing or quiescent. Because tissue stem cells were thought to be relatively dormant to evade DNA damage or telomere shortening during DNA replication, these findings led later investigators to assume that +4 position "label-retaining cells" were the ISCs.

Direct evidence that CBCs were in fact ISCs remained elusive until 2007, when Barker et al. (2007) using an *Lgr5-EGFP-IRES-creER^{T2}* knock-in transgenic mouse lineage tracing approach, reported that CBC cells exclusively express the *Lgr5* gene, and these Lgr5$^+$ CBCs generated all types of differentiated intestinal epithelial cells. Lgr5$^+$ stem cells divide every 24 h, giving rise to progeny called "transit-amplifying cells" (TA cells) that reside just above the crypt stem cell zone. TA cells divide vigorously, generating 16–32 differentiated cells daily. Differentiated epithelial cells are pushed out along the crypt–villus axis toward the tip of the villus, before eventually being sloughed off into the gut lumen 4–5 days later (**Figure 1**).

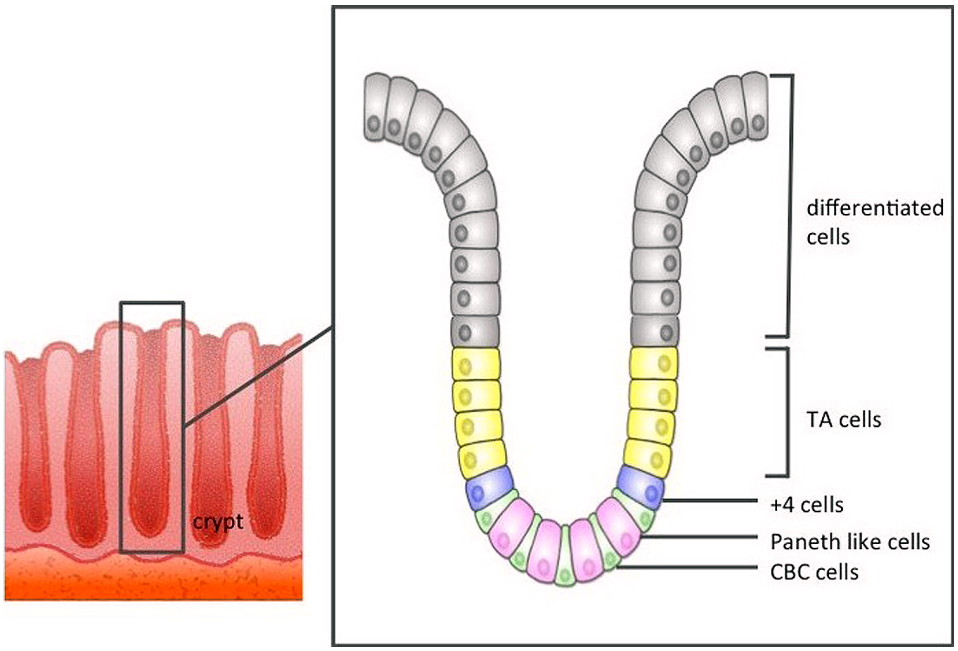

FIGURE 1 | The structure of the large intestine crypt. Each crypt comprises crypt base columnar cells (CBC cells) at the bottom, and these CBC cells are driven toward the lumen as they differentiate.

Similar lineage tracing studies using genes expressed in quiescent +4 cells (*Bmi1*, *mTERT*, *HOPX*, and *Lrig1*) have shown that these cells can also yield all intestinal epithelial lineages (Sangiorgi and Capecchi, 2008; Montgomery et al., 2011; Takeda et al., 2011; Powell et al., 2012). This led to the idea that quiescent +4 cells may revert back to robustly dividing Lgr5^{+} stem cells upon crypt damage, thus acting as an ISC reservoir (Tian et al., 2011). Although this explanation may account for the co-existence of active Lgr5^{+} cells and quiescent +4 cells with ISC capabilities, it has since been shown that genes expressed in +4 cells are also expressed in Lgr5^{+} cells and differentiated intestinal cells (Munoz et al., 2012).

Interestingly, secretory progenitor cells that redundantly express the Notch ligand delta-like1 (Dll1) have been shown to revert to Lgr5^{+} stem cells upon intestinal damage (van Es et al., 2012). More recently, a fraction of Lgr5^{+} cells were identified as the label-retaining cells (LRC) and were shown to be committed to differentiate into Paneth cells (Buczacki et al., 2013). Buczacki et al. (2013) using an elegant lineage tracing strategy, demonstrated that these Lgr5^{+} LRCs formed clonal crypt structure after intestinal damage. Although Lgr5^{+} LRCs and Dll1^{high} cells are not identical in location or Lgr5 expression, these studies indicate plasticity between the secretory progenitors and ISCs and that reserve pools may exist that can regain stem cell signatures upon crypt damage.

CELLS OF ORIGIN IN COLORECTAL NEOPLASMS

Terminally differentiated intestinal cells are post-mitotic and have a lifespan of 4–5 days before being shed into the gut. This short-lived fate is irreversible and renders it unlikely that they would accumulate a sufficient number of "driver" mutations for neoplastic growth, especially considering that mutagenesis in human cells is a rare event (Drake et al., 1998). In contrast, ISCs are the only long-living cells in the intestinal epithelium and are thus more plausible candidate cells of origin for intestinal tumors (**Figure 2**).

Indeed, recent studies have demonstrated that Lgr5^{+} cells may also function as stem cells within intestinal adenomas (Barker et al., 2009). Barker et al. (2009) crossed *Lgr5-EGFP-IRES-creER*^{T2} knock-in mice with *APC*$^{flox/flox}$ mice to produce an Lgr5^{+} stem

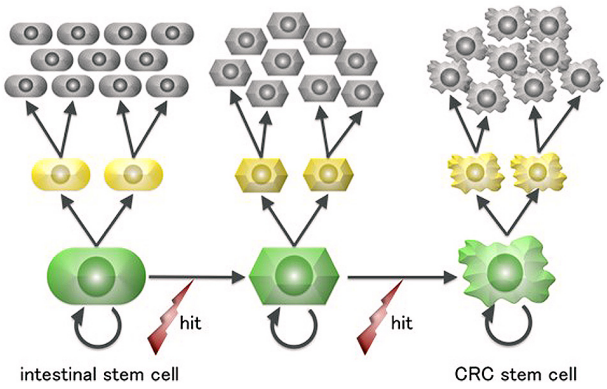

FIGURE 2 | A scheme for colorectal cancer stem cell generation from intestinal stem cells. Intestinal cells are the only long-lived cells in the human large intestine epithelium and thus can undergo the multiple mutagenic events required for neoplastic transformation.

cell-specific knockout of *APC*, which resulted in the formation of macroscopic adenomas. In contrast, upon deletion of *APC* in TA or differentiated intestinal cells, these cells only formed microscopic adenomas. These data suggest that $Lgr5^+$ stem cells, but not their differentiated progeny, are potential cells of origin of intestinal adenoma.

IDENTIFYING COLORECTAL CANCER STEM CELLS

Cancer stem cells are defined as the cancer cells that drive tumorigenesis through long-term self-renewal and production of differentiated, non-tumorigenic progenies. The present gold standard for defining CSC "stemness" is to show their capacity to transfer disease into immunodeficient mice at limiting dilutions. This xenograft assay involves fluorescence-activated cell sorting (FACS) of single cancer cells that exhibit the putative CSC cell signature and subsequent quantification of their ability to develop tumors resembling the original tumor. While this assay represents the only methodology presently available, it is important to consider its limitations when interpreting the resultant data. First, the CSC markers that have been used to date only enrich, to various degrees, the CSC fraction within the population; they do not permit complete discrimination between the CSC and non-CSC pools. Second, differences between the tumor microenvironment of the original site and the transplanted recipient may impact CSC function (Bissell and Labarge, 2005). Growth factors or hormones essential for the tumor growth may be absent, or growth may be attenuated due to the species barrier between rodents and humans.

A major focus for CSC research has been the identification of surrogate markers that distinguish CSCs from non-CSCs within the tumor bulk. With respect to CRC, prominin-1 (CD133) was initially used as a putative CRC stem cell marker. CD133-positive cells derived from human CRCs generated tumors histologically identical to the original tumors in the xenograft assay, whereas CD133-negative cells showed reduced tumor initiation (O'Brien et al., 2007). However, this finding was contested by other studies demonstrating that CD133-negative cells propagated tumors as well (Dalerba et al., 2007; Shmelkov et al., 2008). Sorting by other surface markers, such as CD44 (Dalerba et al., 2007), CD166 (Levin et al., 2010), and ALDH1 (Huang et al., 2009), and by a combination of such markers was employed to isolate CRC stem cells in later studies. More specific markers of ISCs, such as LGR5 or EPHB2, have also been reported to mark the CRC stem cell population (Merlos-Suarez et al., 2011; Kemper et al., 2012). More recently, Schepers et al. (2012) demonstrated genetic lineage tracing of $Lgr5^+$ cells within mouse adenomas, indicating that a small population of cells within the adenoma (5–10%) was responsible for adenoma self-renewal and production of differentiated $Lgr5^-$ adenoma cells. Compared with FACS-based experiments, in which cells are detached from the niche and dissociated into single cells, genetic lineage tracing experiments might provide more physiological results. Genetic tracing experiments using human CRC samples are warranted in future studies.

GENETIC ALTERATIONS IN CRC

Colorectal tumors can be stratified into a number of groups based on their mutational profile, which suggests several distinct routes of colorectal neoplastic formation are possible. One well-established pathway is the multistep genetic carcinogenesis initially proposed by Fearon and Vogelstein (1990). This pathway is referred to as the adenoma to carcinoma sequence, as CRCs arising via this pathway originate from tubular adenomas. In particular, this pathway is triggered by *APC* gene inactivation, which results in ligand-independent Wnt pathway activation, followed by genetic aberrations in various signaling pathways such as *KRAS* in RAS–RAF pathway, *SMAD4* in transforming growth factor beta pathway, *PIK3CA* in AKT-mTOR pathway, and *TP53*. These types of CRCs almost invariably accompany chromosomal aneuploidy or instability of the genome characterized as chromosomal instability (CIN).

Shortly after the proposal of a multistep model for CRC carcinogenesis, subsets of CRCs were shown to carry shorter repetitive DNA elements or microsatellites than normal tissues (Ionov et al., 1993). This signature, microsatellite instability (MSI), marks impairment of the DNA mismatch repair (MMR) system and is observed in CRCs from Lynch syndrome or so called hereditary non-polyposis colon cancer (HNPCC) patients (Peltomaki et al., 1993), as well as in 12–17% of the sporadic CRCs (Ward et al., 2001; Popat et al., 2005). These sporadic CRCs with MSI exhibit clearly different molecular signatures from CIN CRCs: they are near-euploidy or chromosomally stable and are associated with the *BRAF* gene mutation (Rajagopalan et al., 2002). Epigenetic silencing of the MMR genes, mainly *hMLH1*, is often observed (Kane et al., 1997). Further investigations have shown that not only *hMLH1* but also numerous other genes comprising CpG dinucleotide-rich promoter regions are predisposed to epigenetic silencing by promoter methylation (termed the CpG island methylated phenotype, CIMP; Toyota et al., 1999). Serrated polyps of the colon, predominantly microvesicular hyperplastic polyps (MVHPs) and sessile serrated adenoma/polyps (SSA/Ps) were later found to exhibit molecular features similar to those of MSI CRCs (Yang et al., 2004), indicating their potential as the precursors of MSI CRCs. This pathway is referred to as the serrated pathway, arising from serrated polyps to sporadic CRCs with MSI, successively acquiring the *BRAF* mutation, CIMP, and MSI along with tumor development.

Another pathway of colorectal carcinogenesis, the alternative pathway arising via traditional serrated adenomas (TSAs) has also been proposed (Shen et al., 2007). This pathway is associated with *KRAS* mutation, *MGMT* (O^6-methylguanine-DNA methyltransferase) methylation and MSI (Ogino et al., 2007), although the molecular details of this pathway remain elusive.

Recent large-scale sequencing analyses have identified recurrently mutated genes in CRCs. The initial report by Wood et al. (2007) demonstrated that approximately 80 genes are mutated in a typical CRC; however, most of these are neutral, "passenger," mutations, and not more than 15 mutations are responsible for the initiation, progression, or maintenance of the tumor, i.e., are "driver" mutations. The extensive genetic analysis conducted by the Cancer Genome Atlas project identified the frequency and patterns of altered signaling pathways in sporadic CRCs (Cancer Genome Atlas, 2012). In this report, the cases were classified into

two subtypes, non-hypermutated tumors (with a low frequency of gene mutations) and hypermutated tumors (with a high mutation frequency), roughly corresponding to CIN CRCs and MSI CRCs, respectively. Each subtype showed a disparate pattern of genetic mutations, supporting the idea that they arise from discrete pathways from which they arise.

EXPANSION OF INTESTINAL STEM CELLS *EX VIVO*

The long-term culture of non-transformed intestinal cells has previously been unachievable until we established a method that enabled the expansion of murine ISCs *ex vivo* for more than a year (Sato et al., 2009). This method requires laminin-rich Matrigel to provide the cells with scaffolds, along with culture medium containing the growth factors and the hormones necessary to maintain ISCs: R-spondin 1; EGF; and Noggin. R-spondin 1 was later identified as the ligand for Lgr5 and essential for the effective activation of the Wnt signal (Carmon et al., 2011). EGF is associated with intestinal proliferation, and Noggin negatively regulates the BMP signal, which induces crypt differentiation. Under such conditions, ISCs give rise to additional $Lgr5^{+}$ cells as well as differentiated intestinal cells and build three-dimensional cystic crypt–villus structures (organoids), reminiscent of the *in vivo* intestinal epithelium. $Lgr5^{+}$ cells and Paneth cells reside at the bottom of the crypt component, whereas the villus component comprises differentiated intestinal cells. These organoids can be grown from a single sorted $Lgr5^{+}$ stem cell by addition of the Rho kinase inhibitor, confirming the "stemness" of the $Lgr5^{+}$ cells. We later demonstrated that this method could also be applied to human ISCs as well as human colorectal adenomas and adenocarcinomas with modification of the medium content (Sato et al., 2011). The culture medium for human colorectal stem cells requires Wnt3a, a p38 inhibitor and an ALK 4/5/7 inhibitor in addition to the murine small intestine culture condition, while the colorectal tumor organoids can grow in the absence of certain growth factors, depending on the pathway mutations they harbor. Most organoids derived from colorectal neoplasms can grow after the withdrawal of Wnt3a and R-spondin1, consistent with their *APC* mutation. Alternatively, *KRAS* mutation in the organoids renders EGF dispensable.

APPLICATION OF ORGANOIDS TO CSC STUDY AND PERSPECTIVES

A forward genetic approach is essential for functional analysis of the candidate genes involved in CRC stem cell development from ISCs. Genetically engineered mice such as *Lgr5-EGFP-IRES-creER*^{T2}/*APC*$^{flox/flox}$ mice allow the *in situ* observation of tumor generation from normal intestinal epithelium and the kinetics of the stem cells within the tumor (Barker et al., 2009). However, the establishment of genetically engineered strains requires substantial time, effort and cost, especially when handling multiple genes. Clearly, similar approaches in humans are not possible, with the very rare exceptions of patients with certain inherited disorders.

Organoids are amenable to gene overexpression or knockdown by viral infection (Koo et al., 2012), which provide a unique tool to study the phenotypes resulting from the manipulation of gene expression in human ISCs. Furthermore, Schwank et al. (2013) recently demonstrated the application of the CRISPR/Cas9 system for genome targeting in organoids. In this report, the cystic fibrosis transmembrane conductor receptor (CFTR) gene of the intestinal organoids, derived from cystic fibrosis patients, was corrected by homologous recombination via the CRISPR/Cas9 system. A similar methodology can be employed in the context of oncogenes or tumor suppressor genes as well. In summary, the ability to use organoid culture to model the genetic alterations associated with CRC carcinogenesis provides a promising method by which the genetic events involved in CRC stem cell generation can be functionally studied.

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Characterizing the *Retinoblastoma 1* locus: putative elements for *Rb1* regulation by *in silico* analysis

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Limited understanding of the *Rb1* locus hinders genetic and epigenetic analyses of Retinoblastoma, a childhood cancer of the nervous systems. In this study, we used *in silico* tools to investigate and review putative genetic and epigenetic elements of the *Rb1* gene. We report transcription start sites, CpG islands, and regulatory moieties that are likely to influence transcriptional states of this gene. These might contribute genetic and epigenetic information modulating tissue-specific transcripts and expression levels of *Rb1*. The elements we identified include tandem repeats that reside within or next to CpG islands near *Rb1*'s transcriptional start site, and that are likely to be polymorphic among individuals. Our analyses highlight the complexity of this gene and suggest opportunities and limitations for future studies of retinoblastoma, genetic counseling, and the accurate identification of patients at greater risk of developing the malignancy.

Keywords: retinoblastoma, epigenetics, CpG islands, *in silico* analysis

INTRODUCTION

The retinoblastoma gene (*Rb1*) is one of the most widely studied tumor suppressors (Vogelstein and Kinzler, 2004). Retinoblastoma (RB) is a prototype cancer driven in large part by lesions in *Rb1*, a well-defined genetic element and clinical target. Point mutations, deletions, and epigenetic alterations in *Rb1* are also associated with a number of other malignancies (De La Rosa-Velázquez et al., 2007). Recent advances in genomics and epigenomics have made it possible to study RB in novel ways, with approaches combining multiple complementary techniques revealing key genetic and epigenetic steps at the origin of this malignancy (Reis et al., 2012).

Cryptic genetic and epigenetic variation in *Rb1* might contribute variation in the progression and drug response of RB tumors. It is plausible that differential penetrance and variation in the age of onset, which have been observed in patients with hereditary and non-hereditary RB, are attributed to epigenetic regulation of *Rb1* (Kanber et al., 2009). Three CpG islands (CpG106, 42, and 85) potentially involved in regulation of *Rb1* expression have been identified and investigated in detail (Greger et al., 1989). However, uncovering the genetic and epigenetic complexity of the *Rb1* locus remains challenging. This is in part due to a lack of complete understanding of the cis-regulatory elements controlling the expression of the gene. Furthermore, evidence of imprinted expression of *Rb1* suggests that epigenetic mechanisms might play a central role in the regulation of *Rb1* (reviewed in Reis et al., 2012). We expect that comprehensive analyses of the genetic and epigenetic properties of the human *Rb1* gene might reveal new aspects underlying its regulation. In this study, we have characterized a number of features of *Rb1* and presented some potential mechanisms that might be involved in regulation of this gene. Combining the results of several approaches and databanks will promote a better biological understanding of *Rb1*, and contribute toward improved clinical management and counseling of RB patients.

MATERIALS AND METHODS

We combined a set of methods to identify putative functional elements in the *Rb1* locus. Our inferences are based on publicly available databases and re-analyses of experimental data. **Table 1** lists the softwares used in this study. We defined the Genomic Region under Analysis (GRA) as a sequence that spans from 2 kb upstream of annotated Transcription start site (TSS) of *Rb1* to the end of the gene. This was based on previous studies which defined human putative promoter regions as sequences that correspond to −2000 to +1000 bp relative to the TSS (Marino-Ramirez et al., 2004).

RESULTS

EXPRESSION OF *Rb1* AND mRNA ISOFORMS

According to AceView, *Rb1* is expressed at 3.1 times the average gene. The database provides a comprehensive and non-redundant sequence representation of public mRNA sequences, and identified 33 potentially distinct GT-AG introns in *Rb1* (Thierry-Mieg and Thierry-Mieg, 2006). These result in 17 different mRNAs, 10 of which are produced through alternative splicing. There are 3 probable alternative promoters, 3 non-overlapping alternative last exons, and 3 validated alternative polyadenylation sites

Table 1 | Databases and softwares used in this study.

Application	Program/database	Reference/address
Finding mRNA isoforms	Ace view	www.ncbi.nlm.nih.gov/IEB/Research/Acembly/
	UCSC	http://genome.ucsc.edu/
Expression analysis	Ace view	www.ncbi.nlm.nih.gov/IEB/Research/Acembly/
	Affymetrix exon array	http://genome.ucsc.edu/
	GNF Gene Expression Atlas2	
Promoter detection	Hidden Markov Model	UCSC (http://genome.ucsc.edu/)
	CoreBoost_HM Promoter Prediction	UCSC (http://genome.ucsc.edu/)
	Promoter scan	www-bimas.cit.nih.gov/molbio/proscan/
	Promoter2	www.cbs.dtu.dk/services/Promoter
Alternative transcription start sites	DBTSS	http://dbtss.hgc.jp/
	Eponine	UCSC (http://genome.ucsc.edu/)
	SwithGear	UCSC (http://genome.ucsc.edu/)
Detection of CpGIs	UCSC	http://genome.ucsc.edu/
	Bona fide CGIs	http://epigraph.mpi-inf.mpg.de/download/CpG_islands_revisited/
	CpGProD	http://pbil.univ-lyon1.fr/software/cpgprod.html
	CpGcluster	http://bioinfo2.ugr.es/CpGcluster/
	CpG-MI tool	http://bioinfo.hrbmu.edu.cn/cpgmi/
	Weizmann Evolutionary CpG Islands	UCSC (http://genome.ucsc.edu/)
Estimation of the CGI's methylation status	Bona fide CGIs	http://epigraph.mpi-inf.mpg.de/download/CpG_islands_revisited/
Finding repeated sequences	Estimation of repeat variability	http://hulsweb1.cgr.harvard.edu/SERV/
	Repeat masker	http://genome.ucsc.edu/
Inspecting histone marks	UCSC	http://genome.ucsc.edu/
DNase I hypersensitive sites	UCSC	http://genome.ucsc.edu/
Transcription factor binding sites	CisRed	www.cisred.org/
	PReMode	http://genomequebec.mcgill.ca/PReMod/
	ENCODE	UCSC (http://genome.ucsc.edu/)
Prediction of insulator sites	CTCFBSDB	http://insulatordb.uthsc.edu

(http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/). One variant has a supporting clone (NM_000321.2) in Refseq database. According to the UCSC browser, there are three different transcripts, one of which is represented by Refseq (**Figure 1**). Finally, the *GNF Atlas* indicates that *Rb1* is expressed at variable levels across tissues (supplementary Figure 1).

PROMOTERS AND TSSs

Chromatin state segmentation using Hidden Markov Model (HMM) (Pedersen et al., 1996) indicates that at least two promoters might be found in the *Rb1* region. One promoter is near the canonical TSS and another is within one of its introns. According to current annotation, there is a gene named *LPAR* (*P2RY5*) within this intron. Alternative splicing of *LPAR* results in multiple transcript variants. The second active promoter overlaps with TSS of *Rb1* (**Figure 1**). Promoter prediction with *CoreBoost_HM* identifies 4 hits in the GRA (**Figure 1**). *CoreBoost_HM* integrates DNA sequence features with epigenetic information to identify RNA polymerase II core-promoters (Wang et al., 2009). In addition, multiple TSSs were found using *Eponine* and *SwitchGear* (**Figure 1**). "*Eponine*" provides a probabilistic method for detecting TSS, with good specificity and positional accuracy (Down and Hubbard, 2002). "*SwithGear*" describes the location of TSSs throughout the genome along with a confidence measure for each TSS based on experimental evidence (http://genome.ucsc.edu/). Finally, the DBTSS database, which is based on the TSS sequencing method (TSS-Seq), suggests that distinct TSSs might be active in different cell lines (**Table 2**) (Yamashita et al., 2012). Altogether, the results point to alternative promoters and TSSs in the *Rb1* gene.

DETECTION OF CpGIs

According to the UCSC browser searching criteria for CpGIs (traditional method), there were 3 CpG islands (**Figure 2**) in the *Rb1* (CGIs106, 42, and 85). UCSC identifies CpGIs of human genes using three criteria: (1) GC content greater than 50%, (2) length greater than 200 bp, and (3) large ratio between observed

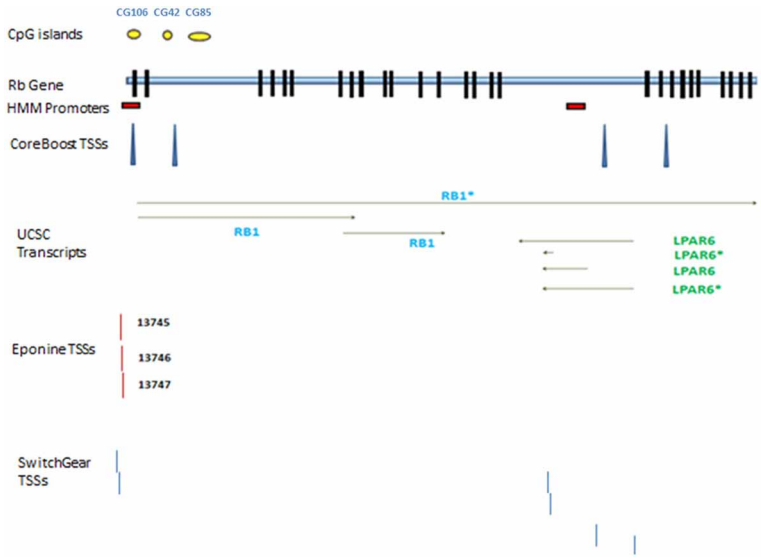

FIGURE 1 | *Rb1* gene structure. Transcribed RNAs from this locus identified by UCSC genome browser. The exons are represented by black blocks. Different promoters and transcription start sites of *Rb1* locus are shown. The diagram shows a schematic representation of results from different databases and programs which are described in the text. The yellow circles show CpG islands identified by UCSC genome browser. There are two red boxes which show the promoters identified by *HMM-Promoter* prediction algorithm. TSSs (Transcription start sites) are recognized by different algorithms such as *CoreBoost*, *Eponine*, and *SwitchGear*. *LPAR* is a gene within *Rb1*.

Table 2 | Transcription start sites (TSSs) identified in the DBTSS database for different cell lines.

Cell line	TSS position (positions are based on UCSC hg19)
Hela	48878016
DLD1	48877884
Beas2B	48877877
Ramos	48877983
	48876242
MCF7	48877937

Table shows the cell lines (left column) and the position of TSSs in the Rb1 gene.

and expected number of CG dinucleotides (Gardiner-Garden and Frommer, 1987). Further analysis indicates additional putative segments containing CpGs. The "*bona fide*" strategy integrates genomic and epigenomic information to screen functional CGIs (Bock et al., 2007). We found eight *bona fide* CpGIs residing within the *Rb1* region (CGI 775-83). Three of them demonstrated positional overlap or neighborhood with three CpGIs predicted by traditional methods and previous studies. Only one of the CpGIs (106 in traditional finding and 775 in Bona fide CGIs) was near the canonical TSS of *Rb1*. The remaining CGIs were in intron 2 (**Figure 2**). Analysis of the targeted genomic region with the "*CpGProD*" program points to different CGIs over the length of *Rb1* (**Figure 2** and **Table 3**). The program investigates prediction of promoter-overlapping CGIs with a longer length and greater CpGo/e ratio compared with non-overlapping start site CGIs (Ponger and Mouchiroud, 2002). Further, the "*CpG cluster*" program detects CpGIs based on the distance between neighboring CpGs. Because a minimum threshold length is not required, *CpG cluster* can find short but fully functional CGIs usually missed by other algorithms. In our study, most of the CpGs identified by this program overlap with the bona fide CGI regions (**Table 3**). Finally, the "Weizmann Evolutionary CpGIs" identified two different CpGIs (CpG2 and 2.6) (**Figure 2**). This custom track of UCSC predicts genome's regulatory elements with highly conserved sequences. **Table 3** shows a comparison of the CpGIs positions identified by different programs.

ESTIMATION OF THE CGI's METHYLATION STATUS

Several programs can be used to predict CGIs methylation status (Carson et al., 2008). The scores reflect the ability of each CGI to maintain its unmethylated state. All genomic CGIs are grouped into four sets: B1(0–0.33), B2(0.33–0.50), B3(0.50–0.67), and B4(0.67–1), whereby CGIs with combined scores >0.5 represent CGIs that are strongly associated with epigenetic regulatory function (http://epigraph.mpi-inf.mpg.de/download/CpG_islands_revisited/). Also, we evaluated two other indicators of methylation status in CGIs: the over-representation of CCGC motif within sequences of CpG islands (Bock et al., 2007) and the presence of H3K4me3 marks in CGIs (Su et al., 2010). We found three CpG islands (CpG775, 779, and 782) within groups B3 and B4. All these CpG islands had CCGC motif in their sequences. Also, we observed other regions which were methylated in different cell lines of ENCODE project (http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr13:48875883-49056026&hgsid=347686961&wgEncodeHaibMethyl450=dense).

TANDEM REPEATS

By using "Estimation of Repeat Variability" toolkit, we found multiple tandem repeats in the GRA (**Table 4**). Three characteristics of the repeats (number of repeated units, unit

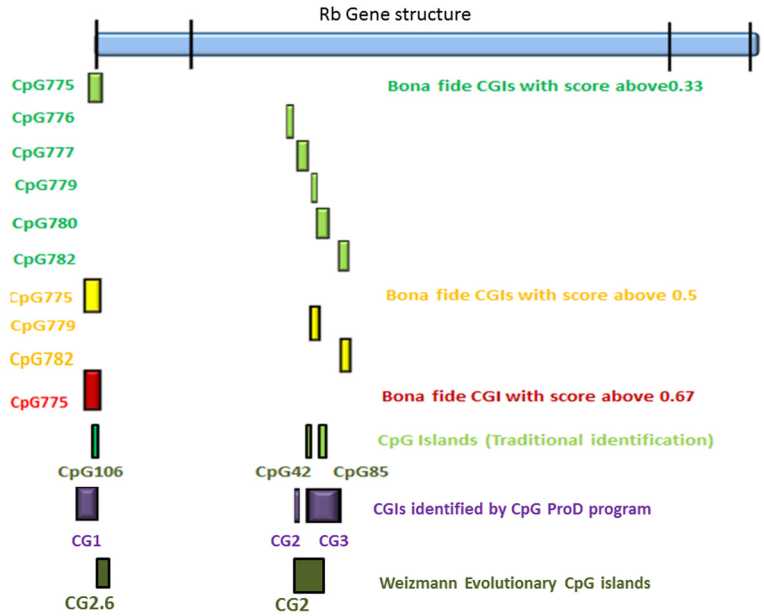

FIGURE 2 | The positions of CpG islands in the *Rb1* locus. The first and last blocks in the schematic gene represent the first and fourth exons of *Rb1*, respectively. "Bona fide" strategy accounts for a number of functional CGIs and estimates their strengths (see scores in the figure). Also, *CpGProD* program predicts promoter-overlapping CGIs. "Weizmann CpG islands" predicts highly conserved CGIs. Although different methods were used, the results are largely concordant.

length, and purity) were considered to produce a numeric "VARscore," which correlates with repeat variability (Legendre et al., 2007). In our result, CGI-775, which includes the TSS of *Rb1* locus, is over a 3 bp unit VNTR. The sequence of this VNTR is: GCCGCCGCCACCGCCGCCGCTGCCGCCGCGGA CCCCCGGCACCGCCGCCGCCGCC. Hence, longer alleles can add CpGs to the number of methylatable sites. Another tandem repeat identified by this software is downstream of CpGI number 6 recognized by CpG cluster. CpGI 6 was not found by bona fide as a functional island, but we observed that the CCGC motif is represented 4 times in the segment that includes CpGI 6 and the VNTR. Also, inspection for transcription factor binding sites in this segment by "TFSearch" software, indicates that there is CREB binding site motif in this region. Enrichment of representation of binding site of this transcription factor characterizes methylation free CpG islands (Tate and Bird, 1993; Sunahori et al., 2009).

INSPECTING HISTONE MARKS

We observed H3K4me1 and H3K4me3 through the annotated core *Rb1* promoter (supplementary Figure 2). The observation was made with data from the ENCODE project. H3K4me1 and H3K4me3 positive marks were mostly mirroring the acetylated histones. It is of note that the regions of histone marks mostly overlapped with CGI-775 and promoters identified by different programs.

DNase I HYPERSENSITIVE SITES (DNase I HS)

We used *DNase Clusters* track in UCSC genome browser. In the *Rb1* promoter, positions of the DNase I HS sites vary depending on cell line assayed. Notably, DNase I HS sites are mostly mapped to CGI_775, which overlaps with CG106. Also, we found that some of these hypersensitive sites are overlapped with or adjacent to other predicted CpGIs.

TRANSCRIPTION FACTOR BINDING SITES

"*CisRed*" and "*PReMode*" databases were used to detect the boundaries of regulatory regions and TFBs motifs distribution. CisRed summarizes conserved sequence motifs identified by genome scale motif discovery, similarity, clustering, co-occurrence, and coexpression calculations (Robertson et al., 2006). The algorithm used in PReMode predicts transcriptional regulatory modules (Ferretti et al., 2007) in which a number of transcription factors can bind and regulate expression of nearby genes (Ben-Tabou De-Leon and Davidson, 2007; Teif, 2010). There were three modules concentrated within or next to CpGs around TSS. Two modules were near the canonical TSS. Finally, the ENCODE results in UCSC point to regions with abundant binding of transcription factors.

INSULATOR SITES

A comprehensive collection of experimentally determined and computationally predicted CTCF binding sites have been curated in the "CTCFBSDB" database (Bao et al., 2008). We observed 6 putative sites for CTCF binding in GRA, two of which are located in CpGI-775 (**Table 5**).

DISCUSSION

Neural progenitor cells dynamically interact with their environment (Jones and Laird, 1999). The expanded two hit hypothesis

Table 3 | Comparison of CpGs identified with different programs.

Regions (kb)	Traditional CpG finding	Bonafide CpGIs (B3 group)	CpGProD	CpGcluster
1–3.5	CGI106: 1578-2619	CGI775:1429-2956	CG1:1370-3076	#1:1540-1710 #2:1759-2619 #3:2673-2898
10–10.5	No	No		#4:10015-10235
12–12.5	No	No		#5:12159-12302
14.5–16	CGI42:15076-15667	No	CG2:14857-15723	#6:15038-15446 #7:15560-15667 #8:15839-16103
16–17	CGI85:16754-17975	CGI779:16336-16550	CG3:16486-20182	#9:16592-16986
17–18		No		#10:17039-17211 #11:17254-17430 #12:17494-17738 #13:17786-17975
18–19	No	No		#14:18458-18645 #15:18807-19080
19–20.5	No	CGI782: 19195-19545		#16:19167-19443 #17:19596-19672 #18:19823-20089
155–165	No	No	CG4:163702-164409	#19:155929-156023 #20:156294-156415 #21:163774-164177

Although each algorithm has its own strategy, there are some concordances between the results. For simplicity, we have divided the Genomic Region under Analysis (GRA) into smaller segments (First column).

Table 4 | Tandem repeats in *Rb1*.

Consensus sequence	Start-end
GCC*	2194–2246
CA**	14974–15038
TG	44625–44668
TG	104353–104389
AGTCATCTTCTACCAAACC TCACCTCCAGCATTGGGGA GCACACTTCAACACG	125368–126744
AAAC	128996–129033
TTCT	158141–158239

Repeats were recognized by the "Estimation of Repeat variability" toolkit and have Var score above 0.5. Positions are relative to the nucleotide in −2 kb of the canonical Rb1 transcription start site.

**Overlapped with CpG # 775.*

***Neighborhood with CpG #6 identified by CpG cluster.*

Table 5 | CTCF binding motifs within the Genomic Region under Analysis.

Motif sequence	Motif start location
CCGGCCTGGAGGGGGTGGTT	1796
GGAACTGCA	2597

The positions are relative to −2 kb of the canonical transcription start site of the Rb1 gene.

proposes that both genetic and epigenetic aberrations are involved in silencing of tumor suppressor genes in cancers such as RB (Jones and Laird, 1999). Studies have shown the role of epigenetic mechanisms in *Rb1* regulation (Reviewed in Reis et al., 2012), but the exact elements and their relation with *cis* regulatory elements already identified as important for *Rb1* expression has remained elusive. Here we used *in silico* analyses and databases to identify and summarize putative regulatory elements that might contribute to *Rb1* regulation. Identification of these elements suggests new venues for understanding *Rb1* expression and its contribution to disease states. The analyses reinforce the notion that a variety of distinct epigenetic and genetic elements are involved in the control of the activity of the human *Rb1* gene.

A study by Greger et al. (1989) was among the first to provide evidence that changes in the methylation of *Rb1* might play a role in the emergence and progression of RB tumors. They found that CpG106, which overlaps the *Rb1* promoter and exon E1, is methylated in some RB cases. Two other CpGs (CpG 42 and 85) were investigated in other studies. Kanber et al. (2009) observed that an alternative transcript of *Rb1* is preferentially expressed from the maternal allele. It seems that imprinted expression

of *Rb1* is linked to a differentially methylated CpG island in intron 2 of this gene (CpG-85) (Kanber et al., 2009). Also, it has been reported that CpG 42 is biallelically methylated, whereas CpG-106 is biallelically unmethylated (Buiting et al., 2010).

We identified additional CpG islands in the *Rb1* locus and sought to assess their epigenetic state by evaluating other data such as co-occurrence of histone modifications, DNAse 1 sensitivity, transcription factor binding sites, and presence of genomic insulators. One possibility is that these genetic and epigenetic features cooperate to fine tune *Rb1* regulation. Our observations highlight two points. First, the *Rb1* locus includes multiple genomic elements exhibiting potential sensitivity to differential DNA methylation and histone modification. Independent tools identified multiple CpG islands in the locus. In spite of differences between softwares, all of them pointed to multiple CpGs, some of which were corroborated by multiple lines of evidence. These are promising targets for downstream functional analysis. Second, repeats occur within or next to some CpG islands. Hence we expect that the methylation status of the *Rb1* regulatory regions in genomes of different individuals might be affected by repeat number variations in nearby sequences. The potential contribution of these regions to the epigenetic regulation of *Rb1* alleles might be worthy of further study. Individual methylation profile might lead to variable expressivity and penetrance in different patients.

Several mammalian genes contain more than a single TSS (Valen et al., 2009) and *Rb1* does not appear to be an exception. Genes with alternative promoters, often display only one promoter with a CGI (Cheong et al., 2006). On the other hand, most of the putative alternative promoters of *Rb1* are distributed in or next to putative CpG islands. Since methylation sensitive regions carry distinctly different information about gene expression and exhibit different sensitivity to regulatory signals, this type of positioning should not be neglected. Besides, DNA methylation appears to play a significant role in differential usage of alternative promoters and be related to functional diversification between CpGI-containing promoters and CpGI-less promoters. Furthermore, chromatin marks and transcription elements such as enhancers or insulators could cause differential expression levels in *Rb1* or even differential usage of the gene's TSSs. The presence of multiple regulatory elements within the locus confers combinatorial control of regulation through which the number of unique expression states can increase (Maston et al., 2006).

The distribution and amount of histone marks like H3K4me1-3 provide a basis for nucleosome positioning in the *Rb1* locus. H3K4me1 is associated with enhancers and DNA regions downstream of TSSs. The H3K4me3 histone mark is associated with promoters that are active or poised to be activated (Karliæ et al., 2010). This histone mark seems to be an indicator of functional CpG islands (Su et al., 2010). We observed an overlap between the regions including this mark and predicted CpGIs (supplementary Figure 2).

It has been reported that DNA methylation correlates with DNase 1 hypersensitivity (Crawford et al., 2006). We found that DNase 1 hypersensitive regions mapped to CGI_775. This CpG island overlaps with the canonical promoter of *Rb1* and this observation is in agreement with studies indicating that regulatory regions in the promoters tend to be DNase sensitive (Crawford et al., 2006). Noteworthy, we observed several CTCF binding sites in the *Rb1* locus. In vertebrates, the transcription regulator CCCTC-binding factor (CTCF) is the only *trans*-acting factor that is a primary part of insulator sequences that block the interaction between enhancers and promoters (Ohlsson et al., 2001). Hence, CTCF is at the core of the machinery that exerts epigenetic control of diverse imprinted loci and participates in promoter activation and repression. Evidence points toward a role for the 11-zinc finger CCCTC-binding factor (CTCF) in the establishment of DNA methylation free zones and the regulation of cell cycle–related genes (Tang et al., 2002; Filippova et al., 2005). CTCF-bound insulators separate transcriptionally active and silent chromatin domains, with their function depending strongly on the local status of DNA methylation and chromatin modifications. It has been suggested that active genes have a DNA fragment with insulator properties and CTCF binding sites in their 5′ ends (Filippova et al., 2005).

Numerous experimental and clinical studies investigate the role of DNA methylation and other epigenetic marks in human diseases (Kanwal and Gupta, 2012). However, in spite of genome-wide patterns, the association between genomic polymorphisms and altered epigenetic status of specific genes is elusive. One interesting possibility is that genetic variations in the *Rb1* gene (including VNTRs) might contribute to the methylation status of the region. Hence, experimental methylation analysis would benefit most if coupled with the sequencing of primary genomic samples. Furthermore, genetic variations in repetitive segments not usually targeted in mutation screens might enable a better understanding of unexpected confounders due to personal genome variation. The proposed set of *Rb1* regulatory elements offers venues to understand the developmental dynamics and individual variation in the expression of the *Rb1* gene. Altogether, we expect that interactions between genetic and epigenetic elements of *Rb1* might cause tissue-specific alternative transcripts, different expression level, and possibly variable penetrance and disease severity in patients with RB.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00002/abstract

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Multilayer-omics analyses of human cancers: exploration of biomarkers and drug targets based on the activities of the International Human Epigenome Consortium

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Epigenetic alterations consisting mainly of DNA methylation alterations and histone modification alterations are frequently observed in cancers associated with chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, or with cigarette smoking. Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and precancerous stages. On the other hand, in patients with cancers, aberrant DNA methylation is frequently associated with tumor aggressiveness and poor patient outcome. Recently, epigenome alterations have been attracting a great deal of attention from researchers who are focusing on not only cancers but also neuronal, immune and metabolic disorders. In order to accurately identify disease-specific epigenome profiles that could be potentially applicable for disease prevention, diagnosis and therapy, strict comparison with standard epigenome profiles of normal tissues is indispensable. However, epigenome mechanisms show heterogeneity among tissues and cell lineages. Therefore, it is not easy to obtain a comprehensive picture of standard epigenome profiles of normal tissues. In 2010, the International Human Epigenome Consortium (IHEC) was established to coordinate the production of reference maps of human epigenomes for key cellular states. In order to gain substantial coverage of the human epigenome, the IHEC has set an ambitious goal to decipher at least 1000 epigenomes within the next 7–10 years. We consider that pathway analysis using genes showing multilayer-omics abnormalities, including genome, epigenome, transcriptome, proteome and metabolome abnormalities, may be useful for elucidating the molecular background of pathogenesis and for exploring possible therapeutic targets for each disease.

Keywords: epigenetics, epigenome, DNA methylation, International Human Epigenome Consortium (IHEC), multilayer/integrated disease omics analyses

MICRO RNAs AND HUMAN DISEASES

The Encyclopedia of DNA Elements (ENCODE) Consortium[1] data have revealed in more detail the high degree of complexity of the mammalian transcriptome: 75% of the genome is transcribed into different types of RNA molecules, e.g., protein-coding, long non-coding, pseudogenes, and small RNA genes (Djebali et al., 2012). RNA molecules show much greater variety than previously suspected. Among such RNA molecules, microRNAs (miRNAs) are non-coding RNAs comprising about 22 nucleotides initially transcribed by RNA polymerase II as primary miRNA (pri-miRNA) molecule precursors that possess a stem loop structure (Jinek and Doudna, 2009). RNase III Drosha acts over pri-mRNAs generating a pre-miRNA containing the hairpin (Jinek and Doudna, 2009). The pre-miRNAs are then exported to the cytoplasm and processed by Dicer into mature miRNAs, which are subsequently translocated into the RNA-induced silencing complex (RISC; Gomes et al., 2013). Each miRNA has multiple tasks, such as transcriptional repression via binding to partially complementary sequences in the 3′-untranslated regions of the target mRNAs and direct mRNA degradation via binding to perfectly complementary sequences (He and Hannon, 2004). Therefore, deregulation of miRNA levels may disturb the expression profiles in cells, thereby playing a key role in induction of diseases, such as cancers, neurodegenerative diseases, and autoimmune diseases.

EPIGENETICS AND miRNAs

Saito et al. revealed that treatment with the DNA demethylating agent 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor 4-phenylbutyric acid induced marked changes in the expression profiles of miRNAs in human cancer cell lines. In particular, DNA hypermethylation and induction of active histone marks in the

Abbreviations: CIMP, CpG island methylator phenotype; COPD, chronic obstructive pulmonary disease; IHEC, International Human Epigenome Consortium; LC, normal lung tissue; LN, non-cancerous lung tissue obtained from patients with lung adenocarcinoma; LT, lung adenocarcinoma tissue; PBAT, post-bisulfite adaptor-tagging; RC, normal renal cortex tissue; RN, non-cancerous renal cortex tissue obtained from patients with clear cell renal cell carcinoma; RT, clear cell renal cell carcinoma tissue.

[1] https://genome.ucsc.edu/ENCODE/

promoter region of miR-127 resulted in decreased and increased expression of miR-127, respectively (Saito et al., 2006). Activation of miR-512-5p by epigenetic treatment induced apoptosis of human gastric cancer cell lines via suppression of the *MCL1* gene (Saito et al., 2009). In the human colon cancer cell line HCT116, disturbance of miRNA expression patterns has been reported after disruption of both *DNA methyltransferase* (*DNMT*) *1* and *DNMT3B* (Lujambio et al., 2007). Findings accumulated to date clearly indicate that expression levels of multiple miRNAs, such as let-7a-3, miR-1, miR-9-1, miR-9-3, miR-34a, mir34a*, mir34b/c, miR-124a, miR-126. miR127, miR-342, and miR-512-5p, are regulated epigenetically (Saito et al., 2013).

On the other hand, the expression of many proteins involved in epigenetics is regulated by miRNAs. For example, miR-152 acts as a tumor suppressor via suppression of *DNMT1* (Huang et al., 2010). The miR-29 family targets *DNMT3A* and *DNMT3B*, whereas miR-101 targets *EZH2* and may alter global chromatin structure (Fabbri et al., 2007). In addition, it has been shown that miRNA has the capacity to recognize chromatin by increasing the methylation of histone, for example through histone H3 lysine 27 tri-methylation (Kim et al., 2008). Thus the close connection between epigenetic alterations and miRNA dysregulation may have a great impact on human diseases.

PARTICIPATION OF EPIGENETIC ALTERATIONS IN MULTISTAGE HUMAN CARCINOGENESIS

Epigenetic alterations, consisting mainly of DNA methylation alterations and histone modification alterations, are often observed in cancers that are associated with chronic inflammation and/or persistent infection with viruses, such as hepatitis B or C viruses, Epstein–Barr virus, and human papillomavirus, or with cigarette smoking (Kanai and Hirohashi, 2007). Accumulating evidence suggests that alterations of DNA methylation are involved even in the early and precancerous stages (Arai and Kanai, 2010). On the other hand, in patients with cancers, aberrant DNA methylation is frequently associated with tumor aggressiveness and poor patient outcome (Kanai, 2008). Precancerous conditions showing alterations of DNA methylation may progress rapidly and generate more malignant cancers (Kanai, 2010).

As we described in the webpage of our laboratory[2], even though genetic alterations, such as activation of oncogenes and inactivation of tumor suppressor genes, have been considered to provide the molecular framework of multistage human carcinogenesis, genetic events alone may not explain the histological heterogeneity underlying the complex biological characteristics of tumors. Therefore, in the 1990s, we began to focus on epigenetic events that can be reversible, in an attempt to explain why cancers show such histopathological heterogeneity. At a time when only two genes, *RB* and *VHL*, were known as tumor suppressor genes silenced by DNA methylation, we showed for the first time that the *CDH1* gene, which encodes the E-cadherin cell adhesion molecule and acts as tumor suppressor, is silenced by DNA methylation around the promoter region in human cancers (Yoshiura et al., 1995). In 1996, we demonstrated that DNA methylation alterations frequently occurred at multiple loci on chromosome 16, one of the hot spots for loss of heterozygosity in liver cancers. This preceded loss of heterozygosity even at the chronic hepatitis or liver cirrhosis stages, which are widely considered to be precancerous conditions. This was one of the earliest reports of aberrant DNA methylation at the precancerous stage (Kanai et al., 1996).

Since then, we have reported DNA methylation alterations in tissue specimens at precancerous stages and in cancers using a candidate-gene approach. As an example of inflammation-associated carcinogenesis, ductal adenocarcinomas of the pancreas frequently develop in a background of chronic pancreatitis. Under these conditions, at least a proportion of peripheral pancreatic duct epithelia may be at the precancerous stage. It has been reported that the average number of methylated tumor-related genes, the incidence of DNA methylation of at least one of such genes, and the expression level of *DNMT1* protein are increased in pancreatic duct epithelia with an inflammatory background, and in another precancerous lesion, pancreatic intraductal neoplasia (PanIN), in comparison with normal pancreatic duct epithelia (Peng et al., 2006).

Urothelial carcinomas of the urinary bladder, renal pelvis, and ureter are clinically remarkable because of their multicentricity and tendency to recur. Such multiplicity may be attributable to the "field effect." Even non-cancerous urothelia showing no marked histological findings from patients with urothelial carcinomas can be considered precancerous, because they may have been exposed to carcinogens in the urine. It has been reported that the average number of methylated tumor-related genes and the expression level of *DNMT1* protein are increased in non-cancerous urothelia showing no marked histological findings from patients with urothelial carcinomas, in comparison with normal urothelia from patients without urothelial carcinomas (Nakagawa et al., 2005). Thus, overexpression of the major DNMT, *DNMT1*, may result in accumulated hypermethylation of DNA for tumor-related genes (Etoh et al., 2004). On the other hand, splicing alteration of *DNMT3B* may induce chromosomal instability through DNA hypomethylation of pericentromeric satellite regions (Saito et al., 2002).

As we described in the webpage of our laboratory[2], after genome-wide epigenetic (epigenome) analysis had become practical, we employed the bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) method for overviewing the DNA methylation tendency of large individual chromosomal regions. Although precancerous conditions in the kidney have rarely been described, despite the lack of any marked histological findings or association with chronic inflammation or persistent infection with pathogens, it can be considered that non-cancerous renal cortex tissue obtained from patients with renal cancers is already at the precancerous stage showing genome-wide DNA methylation alterations (Arai et al., 2006). We showed that DNA methylation profiles at the precancerous stage are inherited by renal cancers developing in individual patients, and that DNA methylation alterations at the precancerous stage determine both the aggressiveness of subsequently developing cancers and patient outcome through inducing further epigenetic and genetic alterations (Arai et al., 2009a). In addition, we have developed indicators for carcinogenetic risk estimation in patients with chronic hepatitis and liver cirrhosis (Arai et al., 2009b), indicators

[2]http://www.ncc.go.jp/en/nccri/divisions/01path/01path01.html

for estimating the risk of development of urothelial carcinomas that can be determined from urine samples (Nishiyama et al., 2010), diagnostic markers of pancreatic cancer that can be assessed from specimens of pancreatic juice (Gotoh et al., 2011), and indicators for prognostication of kidney, liver, pancreas, and urinary bladder cancers based on DNA methylation profiling. Based on these findings, we have filed patent applications for epigenome diagnosis techniques, and are now attempting to apply them practically.

ACTIVITIES OF THE INTERNATIONAL HUMAN EPIGENOME CONSORTIUM (IHEC)

Recently, epigenome alterations have been attracting a great deal of attention from researchers who are focusing on not only cancers but also neuronal, immune, and metabolic disorders. On the basis of epigenome profiling, attempts are now being made to elucidate the molecular pathogenesis of such diseases and to explore possible biomarkers and drug targets. In order to accurately identify such disease-specific epigenome profiles that could be potentially applicable for disease prevention, diagnosis, and therapy, strict comparison with standard epigenome profiles of normal tissues is indispensable. However, epigenome mechanisms show heterogeneity among tissues and cell lineages. Therefore, it is not easy to obtain a comprehensive picture of standard epigenome profiles of normal tissues. Based on improvements in next-generation sequencing technology, international collaboration will likely help to reveal standard epigenome profiles.

In 2010, the IHEC was established by researchers and founding agencies from Canada, South Korea, the EU, Italy, Germany, Japan, and the USA (Bae, 2013). As described in the webpage of IHEC[3], the primary goal of the IHEC is "to coordinate the production of reference maps of human epigenomes for key cellular states that are relevant to health and diseases." In order to achieve substantial coverage of the human epigenome, the IHEC has set an ambitious goal to decipher at least 1000 epigenomes[3]. To attain this goal, IHEC will use robust techniques to generate (1) high-resolution maps of histone modifications, H3K4me3, H3K9me3, H3K27me3, H3K27ac, H3K4me1, and H3K36me3, (2) high-resolution DNA methylation maps, (3) landmark maps of transcription start sites for all protein-encoding genes, and (4) a comprehensive catalog of non-coding and small RNAs and their patterns of expression[3]. The target cell types being studied by each team in the participating countries are shown on the IHEC website[4].

In Japan, three Japanese IHEC teams[5] including our team are supported by the Core Research for Evolutional Science and Technology division of the Japan Science and Technology Agency. To strengthen the research bases for cancers of digestive organs, including hepatocellular carcinomas and gastric carcinomas, which show high incidences in Japan, we are now performing standard epigenome analyses of normal epithelial cell lineages in digestive organs (**Figure 1**). Target cells of sufficient quality and quantity are being obtained from materials surgically resected from a range of Japanese patients. For example, for liver, we have obtained samples of normal liver tissue distant from sites of liver metastases from primary colon cancers in partial hepatectomy specimens from patients without viral hepatitis, chronic hepatitis, or liver cirrhosis. To isolate hepatocytes, we have performed collagenase perfusion of cannulated branches of the hepatic vein, followed by low-velocity centrifugation. On average, more than 10^7 dispersed cells can be obtained from each case, and immunocytochemistry has confirmed that the hepatocytes are more than 95% pure. In the stomach and colorectum, we initially employ the crypt isolation technique and collagenase digestion. Thereafter, each normal cell lineage is purified by fluorescence activated cell sorting using appropriate antibodies.

Members of our IHEC team have originally developed the post-bisulfite adaptor-tagging method (PBAT), which is an efficient library preparation method for whole-genome bisulfite sequencing (Miura et al., 2012). For the PBAT method, we first perform bisulfite modification followed by adaptor ligation using random priming. The PBAT method minimally requires sub-microgram DNA for mammalian whole-genome bisulfite sequencing without global PCR amplification. A good correlation of the DNA methylation pattern was observed among PBAT, the standard Methyl C-seq methodology developed by Lister et al. (2008), and the Illumina beads chip Infinium assay. The PBAT method is advantageous in that it requires only a small amount of genomic DNA but has good coverage of GC-rich regions, especially in CpG islands and gene-rich chromosomes. We now propose to make the PBAT method one of the standard protocols for IHEC. Under the supervision of the IHEC, we intend to disclose the data we obtain through the National Bioscience Database Center supported by the Japan Science and Technology Agency. Accurate standard epigenome profiles of digestive organ epithelial cells obtained through IHEC activities will be used to explore more useful biomarkers and drug targets of digestive organ cancers.

MULTILAYER/INTEGRATIVE DISEASE OMICS ANALYSES FOR EXPLORATION OF BIOMARKERS AND DRUG TARGETS

Recently big data analysis has impacted various fields of bioscience, especially disease research. It may not be appropriate to perform epigenome analysis including miRNA analysis using clinical samples. Instead, simultaneous multilayer/integrative disease omics analyses would seem more appropriate, including genome, epigenome, transcriptome, proteome, and metabolome analyses for exploration of drug targets. Since 2010, researchers at six National Centers in Japan, i.e., the National Cancer Center, National Cerebral and Cardiovascular Center, National Center for Neurology and Psychiatry, National Center for Global Health and Medicine, National Center for Child Health and Development and National Center for Geriatrics and Gerontology, have been engaged in a research project "Comprehensive exploration of drug targets based on multilayer/integrative disease omics analyses" supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio) (**Figure 2**). This project has been divided among a number of centers specializing in genome, epigenome, transcriptome, proteome, and metabolome analyses of tissue specimens from patients with various diseases that show a high incidence in the Japanese population. Tissue and body fluid

[3]http://ihec-epigenomes.org/about/objectives/

[4]http://ihec-epigenomes.org/research/cell-types/

[5]http://crest-ihec.jp/

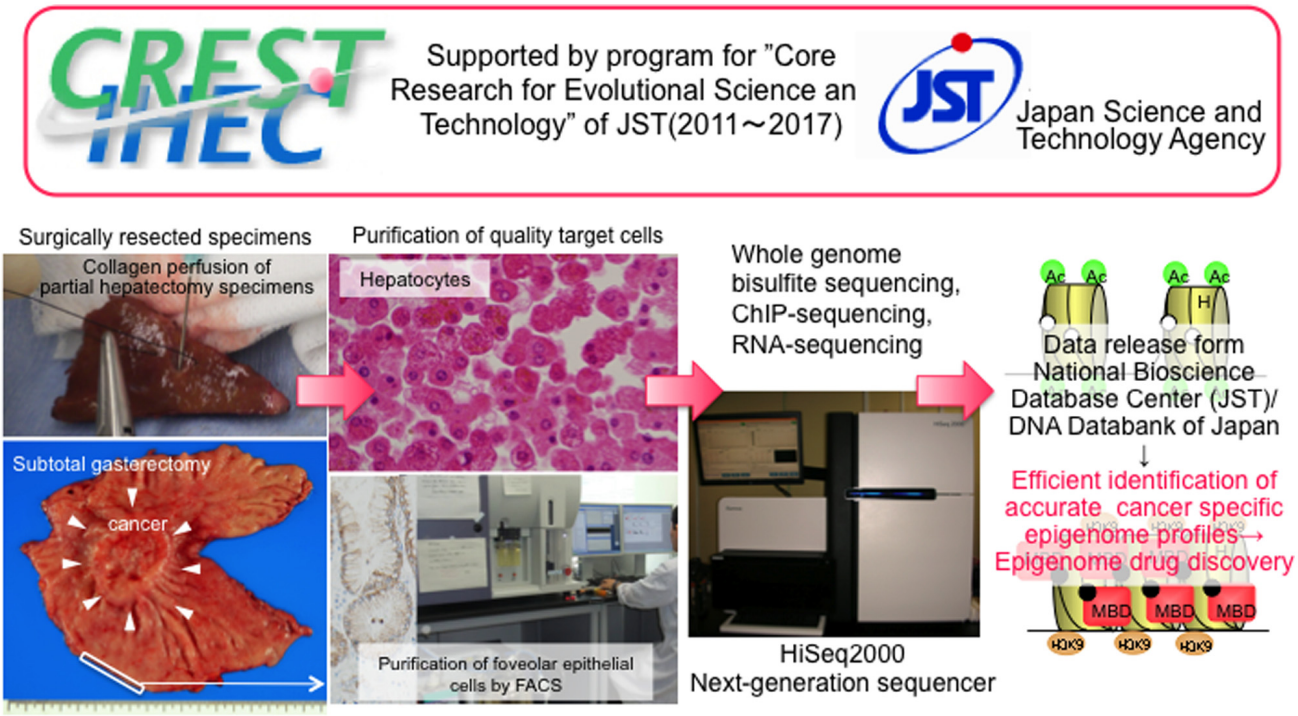

FIGURE 1 | Activity of our team, one of the Japan Teams of the International Human Epigenome Consortium supported by the Core Research for Evolutional Science and Technology division of the Japan Science and Technology Agency (modified from the webpage of our laboratory [http://www.ncc.go.jp/en/nccri/divisions/01path/01path01.html]). We are now performing whole-genome bisulfite sequencing using the post-bisulfite adaptor-tagging method, chromatin immunoprecipitation-sequencing, and RNA-sequencing of purified target cells, i.e., hepatocytes and other live cell lineages, foveolar epithelial cells, and other gastric epithelial cell lineages, and absorptive epithelial cells from the ascending and descending colon and rectum. Accurate epigenome profiling of normal cells will allow the identification of disease-specific epigenome profiles, thus facilitating a potential breakthrough in the prevention, diagnosis, and therapy of diseases.

specimens, cultured cells and animal models of adult cancers, infant leukemia, allergic disease, dilated cardiomyopathy, aortic aneurysm, epilepsy, obesity, non-alcoholic steatohepatitis, spinal canal stenosis, and Alzheimer's disease have been subjected to multilayer-omics analyses. As we described in the webpage of our laboratory[2], we are especially focusing on molecules or molecular pathways which are impaired as a result of multiple mechanisms, such as events in all five omics layers, which may participate in the molecular pathogeneses of diseases and might become potential biomarkers and/or druggable targets (**Figure 2**).

With regard to epigenome analysis of adult cancers in this research project, 414 lung tissue specimens including normal lung tissue (LC) obtained from patients without any primary lung tumor, non-cancerous lung tissue (LN) obtained from patients with lung adenocarcinomas, and lung adenocarcinoma tissue (LT) itself have been subjected to single-CpG resolution Infinium assay. DNA methylation alterations on many probes were evident in LN samples relative to LC samples, and were inherited by, or strengthened in, LT samples. Unsupervised hierarchical clustering using DNA methylation levels in LN samples subclustered patients into clusters I, II, and III. Lung adenocarcinomas in cluster I developed from an inflammatory background in chronic obstructive pulmonary disease (COPD) in heavy smokers, and were locally invasive. Most patients in cluster II were non-smokers and had a favorable outcome. Lung adenocarcinomas in cluster III were most aggressive cancers in light smokers that developed before accumulation of the long-term effects of cigarette smoking, and were probably due to the direct actions of carcinogens, rather than the effects of inflammation. DNA methylation profiles reflecting carcinogenetic factors such as smoking and COPD appear to be established in LNs and may determine the aggressiveness of tumors developing in individual patients, and thus patient outcome (Sato et al., 2014). Among the genes for which DNA methylation status in LN samples was significantly correlated with recurrence of lung adenocarcinomas in individual patients, we focused on *ADCY5, EVX1*, and other genes that were involved in apoptosis and cell adhesion. The mRNA expression levels of these genes were directly regulated by DNA methylation, and a decrease of their mRNA expression in LT samples was significantly correlated with tumor aggressiveness (Sato et al., 2013). When these genes were ectopically expressed in lung cancer cell lines, growth suppression, and apoptosis were induced, indicating that these genes could become therapeutic targets of lung adenocarcinomas.

With regard to epigenome analysis during renal carcinogenesis, 245 renal tissue specimens including normal renal cortex tissue (RC) obtained from patients without any primary renal cancer, non-cancerous renal cortex tissue (RN) obtained from patients with clear cell renal cell carcinomas, and clear cell

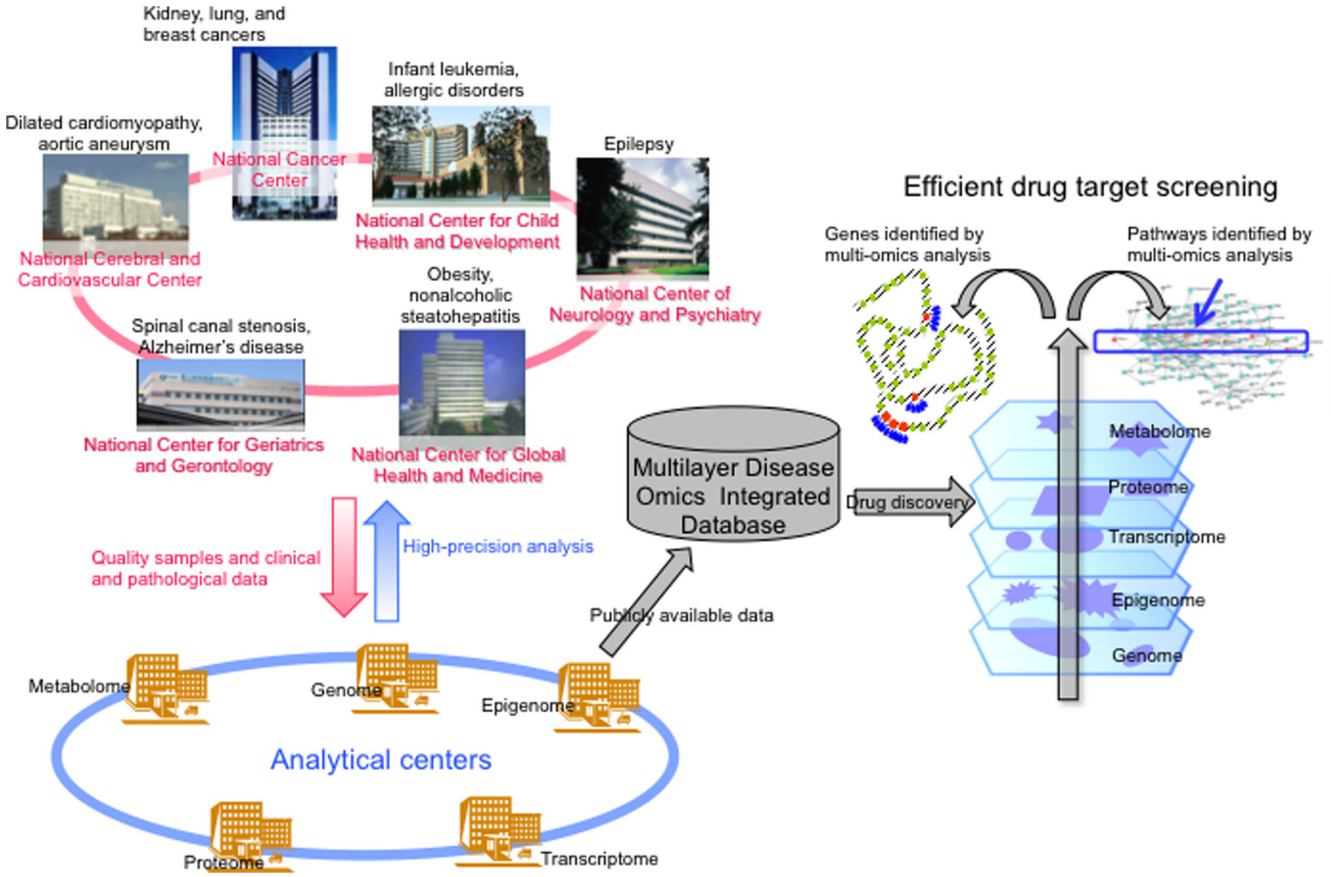

FIGURE 2 | Brief overview of the "Comprehensive exploration of drug targets based on multilayer/integrative disease omics analyses" project supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (modified from the webpage of our laboratory [http://www.ncc.go.jp/en/nccri/divisions/01path/01path01.html]). In this project, researchers in several National Centers in Japan have been split up into analytical centers for genome, epigenome, transcriptome, proteome, and metabolome. We have been focusing on molecules or molecular pathways that are impaired by multiple mechanisms, such as genome and epigenome events, or events in all five omics layers, which may participate in the molecular pathogeneses of those diseases and might become drug targets.

renal cell carcinoma tissue (RT) itself were subjected to the Infinium assay. DNA methylation levels at multiple Infinium probe sites were already altered in RN samples relative to RC samples. Unsupervised hierarchical clustering analysis based on DNA methylation levels at the CpG sites where DNA methylation alterations had occurred even in RN samples and were inherited by, and strengthened in, RT samples divided the clear cell renal cell carcinomas into CpG island methylator phenotype (CIMP)-positive and -negative clusters (Arai et al., 2012). Clinicopathologically aggressive cancers were accumulated in the CIMP-positive cluster, where the cancer-free and overall survival rates of the patients were significantly lower than in the CIMP-negative cluster. *FAM150A, GRM6, ZNF540, ZFP42, ZNF154, RIMS4, PCDHAC1, KHDRBS2, ASCL2, KCNQ1, PRAC, WNT3A, TRH, FAM78A, ZNF671, SLC13A5*, and *NKX6-2* have been identified as renal cell carcinoma-specific CIMP marker genes (Arai et al., 2012). Since CIMP-positive renal cell carcinomas show tumor aggressiveness and poorer patient outcome, we established criteria for prognostication of patients with clear cell renal cell carcinomas using renal cell carcinoma-specific CIMP marker genes. We are now performing pathway analysis based on a Bayesian estimation model using multiple genes showing frequent mutations and alterations of expression at the mRNA, miRNA, and protein levels based on multilayer-omics analyses in each of the CIMP-negative and CIMP-positive renal cell carcinomas for exploration of possible drug targets.

PERSPECTIVES

Once DNA methylation alterations occur during multistage carcinogenesis, such alterations are stably preserved on DNA double strands through maintenance methylation mechanisms by *DNMT1*. Therefore, stable stratification of cancers reflecting clinicopathological diversity may be possible based on epigenome profiling. Genes showing epigenome alterations, such as CIMP-marker genes, may become excellent biomarkers discriminating each tumor type stratified on the basis of epigenome profiling. We consider that pathway analysis using genes showing multilayer-omics abnormalities after stratification based on epigenome profiling may be useful for elucidating the molecular background of carcinogenetic pathways

and for exploring possible therapeutic targets for each tumor type.

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Non-coding RNAs as epigenetic regulator of glioma stem-like cell differentiation

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Glioblastomas show heterogeneous histological features. These distinct phenotypic states are thought to be associated with the presence of glioma stem cells (GSCs), which are highly tumorigenic and self-renewing sub-population of tumor cells that have different functional characteristics. Differentiation of GSCs may be regulated by multi-tiered epigenetic mechanisms that orchestrate the expression of thousands of genes. One such regulatory mechanism involves functional non-coding RNAs (ncRNAs), such as microRNAs (miRNAs); a large number of ncRNAs have been identified and shown to regulate the expression of genes associated with cell differentiation programs. Given the roles of miRNAs in cell differentiation, it is possible they are involved in the regulation of gene expression networks in GSCs that are important for the maintenance of the pluripotent state and for directing differentiation. Here, we review recent findings on ncRNAs associated with GSC differentiation and discuss how these ncRNAs contribute to the establishment of tissue heterogeneity during glioblastoma tumor formation.

Keywords: epigenetics, glioma, cancer stem cells, long non-coding RNA, micro RNA

INTRODUCTION

Gliomas are the most common type of malignant primary brain tumor with an incidence of ∼5 cases per 100,000 persons (Wen and Kesari, 2008). Glioblastoma multiforme (GBM) is the highest grade glioma (grade 4). Despite advances in treatment using combinations of surgery, radiotherapy, and chemotherapy, GBM confers an average life expectancy of around 14 months from diagnosis (Wen and Kesari, 2008). Accumulating evidence indicates that the presence of a subset of cells with the potential to initiate and maintain growth of gliomas might be crucial for their resistance to conventional therapies (Hadjipanayis and Van Meir, 2009). These cells are designated as glioma stem cells (GSCs; Galli et al., 2004; Singh et al., 2004; Lee et al., 2006; Penuelas et al., 2009; Mazzoleni et al., 2010). GSCs and normal neural stem cells appear to share common features including self-renewal and the capability of differentiating into multiple lineages. Intriguingly, recent studies revealed that in addition to GSCs differentiating into non-GSCs, the reverse process might also occur (Gupta et al., 2011; Natsume et al., 2013). This phenotypic plasticity between the GSC and non-GSC states may be regulated by signals within the tumor microenvironment.

Microenvironmental signals, such as sonic Hedgehog (SHH), Wnt, and Notch, have been shown to regulate the properties of cancer stem cells (Reya and Clevers, 2005; Fan et al., 2010; Takebe et al., 2011). SHH has a critical role in the maintenance of GSCs by regulating so-called "stemness" genes and has also been found to be activated in many high-grade gliomas (Clement et al., 2007; Takezaki et al., 2011). The Wnt/β-catenin pathway has been implicated in the role of GSCs in gliomagenesis through tumor proliferation and invasion (Nager et al., 2012). Notch signaling has been shown to promote GSC self-renewal and to suppress GSC differentiation (Shih and Holland, 2006; Fan et al., 2010; Hu et al., 2011). Genes in the receptor tyrosine kinase (RTK) family mediate several oncogenic growth factor pathways, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), that have been linked to malignancy, angiogenesis, self-renewal, and multipotency. Recently, it was shown that constitutively activated EGFRvIII expression and loss of the phosphatase and tensin (PTEN) protein in murine neural stem cells results in the formation of glial tumors (Li et al., 2009a). PDGF overexpression has also been implicated in gliomagenesis, and PDGFs can inhibit glial cell differentiation (Fomchenko and Holland, 2007).

Recent advanced technology to identify non-coding RNAs using microarrays or next generation sequencing technologies provide extraordinary abundance of novel data in genome wide-scale and revealed deeper insights into the biology of non-cording RNAs (ncRNAs). More than 90% of the human genome appears to be transcribed and transcription is not limited to protein-coding regions (Birney et al., 2007). Some ncRNAs may play key regulatory and functional roles. Indeed, significant numbers of ncRNAs, such as microRNAs (miRNAs, miRs) and long non-coding RNAs (lncRNAs), are regulated during development in a cell-type specific manner, and are associated with multiple cell functions (Kapranov et al., 2007). miRNAs are the short non-coding endogenous RNAs that post-transcriptionally regulate the expression of a large number of genes (Bartel, 2004). miRNAs play important roles in a wide variety of physiological and pathological processes including tumor formation. Aberrant expression of miRNA can induce tumor suppression or can have an oncogenic effect resulting in tumor formation (Medina and Slack, 2008; Gangaraju and Lin, 2009). lncRNAs are functional ncRNAs that are potentially key regulators not only of cellular differentiation and proliferation, but may also have tumor suppressive

or oncogenic functions in many types of cancer (Esteller, 2011; Wapinski and Chang, 2011; Hu et al., 2012; Zhang et al., 2013).

In this review, we provide a summary of the current understanding of miRNAs and lncRNAs in gliomas with a focus on their roles in GSCs.

miRNAs IN GSC DIFFERENTIATION

miRNAs are short sequences of 17–25 nucleotides that are not transcribed but have a regulatory function. An RNase III enzyme converts pri-miRNA into pre-miRNA hairpin transcripts that are processed into mature miRNAs and incorporated into a ribonucleoprotein complex called the RNA-induced silencing complex (RISC). The RISC and associated mature miRNA then binds to mRNA and causes a physical block to translation (Ambros and Lee, 2004; Bartel, 2004). Many miRNAs form imperfectly complementary stem-loop structures on the sense strand of the target mRNA. Thus, each miRNA can target multiple mRNA species through recognition of complementary sequences. Upregulation of mature miRNAs may occur as a consequence of transcriptional activation or amplification of the corresponding pre-miRNA locus, whereas downregulation of miRNAs may result from epigenetic silencing or deletion of the corresponding region (Schickel et al., 2008). Although dysregulation of the miRNA-mRNA network has been reported in glioblastoma, little attention has so far been paid to its role in GSCs (Godlewski et al., 2010a). In this section, we describe the information available on the significance of miRNAs in GSCs (**Table 1**).

miR-17-92 CLUSTER

The miR-17-92 cluster is thought to be involved in the regulation of GSC differentiation, apoptosis, and proliferation (Ernst et al., 2010). The level of transcripts from miR-17-92 clusters are significantly higher in primary astrocytic tumors than in normal brain tissues and increase significantly with tumor grade progression. A High-level amplification of the miR-17-92 locus has also been found in glioblastoma specimens. Inhibition of miR-17-92 induces apoptosis and decreases cell proliferation in GSCs. mir-17-92 inhibition is also associated with induction of cyclin-dependent kinase inhibitor 1A (*CDKN1A*), E2F transcription factor 1 (*E2F1*), *PTEN*, and connective tissue growth factor (*CTGF*). Of these, the CTGF gene was shown to be a direct target of miR-17-92 in GSCs.

When GSCs are exposed to the differentiation-promoting conditions, downregulation of the oncogenic miR-17-92 cluster is directly related to the concomitant upregulation of CTGF (Ernst et al., 2010).

miR-124 AND miR-137

The initial analysis of miR-124 showed that it promotes neuronal differentiation by targeting the polypyrimidine tract-binding protein 1 (PTBP1) that encodes a global repressor of alternative pre-mRNA splicing; miR reduces the level of PTBP1, which results in an increase in the production of nervous system-specific alternative RNA splicing and promotes the differentiation of progenitor cells to mature neurons (Makeyev et al., 2007). Subsequent analysis showed that both miR-124 and miR-137 are downregulated in high-grade gliomas and up-regulated during adult neural stem cell differentiation (Silber et al., 2008). Transfection of miR-124 or miR-137 inhibits proliferation of GSCs, via suppression of cyclin-dependent protein kinase 6 (*CDK6*), and induces morphological changes in human GSCs and expression of neuronal differentiation markers. Overexpression of miR-124 has consistently been found to inhibit the CD133+ cell subpopulation of the neurosphere and to downregulate stem cell markers, such as *BMI1*, *Nanog*, and *Nestin*. These effects could be rescued by re-expression of *SNAI2*, another direct target of miR-124 (Xia et al., 2012).

miR-451

Analysis of the miRNA profiles of GSC (CD133+ cells) and non-GSC (CD133– cells) populations showed that several miRNAs, including miR-451, miR-486, and miR-425, are upregulated in CD133– cells. Transfection of cells with miR-451 has been shown to induce disruption of glioblastoma neurospheres (Gal et al., 2008). Interestingly, this study also showed that SMAD

Table 1 | List of miRNAs dysregulated in GSCs.

MicroRNAs	Direct targets	Roles in GSC	Reference
miR-17-92 cluster	*CTGF*	Differentiation (–), proliferation (+), apoptosis (–)	Ernst et al. (2010)
miR-451	*CAB39*	Differentiation (–), proliferation (+), apoptosis (–)	Godlewski et al. (2010b)
miR-1275	*CLDN11*	Differentiation (–), proliferation (+)	Katsushima et al. (2012)
miR-138	*CASP3, BLCAP, MXD1*	Differentiation (–), proliferation (+), apoptosis (–)	Chan et al. (2012)
miR-137	*CDK6*	Differentiation (+), proliferation (–)	Silber et al. (2008)
miR-34a	*MET, NOTCH1, NOTCH2, CDK6*	Differentiation (+), proliferation (–), apoptosis (+)	Li et al. (2009b), Guessous et al. (2010)
miR-302-367 cluster	*CXCR4*	Differentiation (+), proliferation (–), invasion (–)	Fareh et al. (2012)
miR-124	*SNAI2*	Differentiation (+), proliferation (–), invasion (–)	Xia et al. (2012)
miR-204	*SOX4, EPHB2*	Differentiation (+), proliferation (–), invasion (–)	Ying et al. (2013)
miR-128	*BMI1, SUZ12*	Differentiation (+), proliferation (–), radiosensitivity (–)	Godlewski et al. (2008), Peruzzi et al. (2013)

(+) = increased, (–) = decreased.

proteins, which are associated with GSC regulation, can upregulate miR-451 by binding to its promoter region. Thus, there is a link between miRNAs and well-known stem cell regulating proteins (Piccirillo et al., 2006). Another interesting finding regarding miR-451 is that its expression level is correlated with glucose concentration. High glucose levels are associated with relatively high levels of miR-451 expression, which promote cell growth; miR-451 expression levels decrease under low glucose conditions, resulting in a reduced rate of cell proliferation but an enhanced rate of cell migration and survival in glioblastomas. This miR-451 effect is mediated by liver kinase B1 (*LKB1*). These data indicate that tumor cells can survive under metabolic stress conditions and also seek out locations with more favorable growth conditions by migration influenced through an LKB1/AMPK pathway mediated by miR-451 (Godlewski et al., 2010b).

miR-34a

miR-34a is tumor-suppressive and is downregulated in human glioma tissues; miR-34a directly inhibits the expression of *c-Met*, *Notch-1,* and *Notch-2* in GSCs (Li et al., 2009b). Notch is a critical regulator of cell-fate during development and also of normal stem cell maintenance (Fan et al., 2006; Shih and Holland, 2006; Fan et al., 2010). Activation of the Notch pathway enhances the stemness, proliferation, and radioresistance of GSCs (Wang et al., 2010). Ectopic expression of miR-34a in glioma cells inhibits cell proliferation, survival, and migration. In addition, miR-34a induces GSC differentiation as evidenced by the decreased expression of stem cell markers and increased expression of differentiation markers (Guessous et al., 2010).

miR-128

Two studies have described a link between miR-128 and the polycomb repressor complex (PRC). Two major complexes, PRC1 and PRC2, are recognized as key epigenetic regulators during development (Lund and van Lohuizen, 2004) and are required for maintaining self-renewal and multi-potential capability (Richly et al., 2011). The first study demonstrated that miR-128 has a tumor-suppressive function and that this is downregulated in glioblastoma tissue. miR-128 expression significantly reduces glioma cell proliferation both *in vitro* and *in vivo* via downregulation of the oncogene *Bmi-1* that is a component of PRC1. In addition, miR-128 inhibits GSC self-renewal (Godlewski et al., 2008). The second study showed that miR-128 directly targets *SUZ12*, a key component of PRC2. Ectopic expression of miR-128 in GSCs significantly increases their radiosensitivity (Peruzzi et al., 2013). The PRC has been shown to promote normal and cancer stem cell self-renewal and is also implicated in GSC regulation (Abdouh et al., 2009; Suva et al., 2009; Natsume et al., 2013). The findings of these various studies therefore indicate that miR-128 mediates an important epigenetic regulatory pathway in GSCs.

OTHER miRNAs

Several other miRNAs have been implicated in glioma malignancy. Ectopic expression of the miR-302-367 cluster in GSCs inhibits the CXCR4 pathway resulting in the suppression of stemness signatures, self-renewal, and cell infiltration. Inhibition of the CXCR4 pathway leads to the disruption of the SHH-GLI-NANOG network, which is important for cell self-renewal and tumorigenic properties (Fareh et al., 2012). In both GSCs and non-GSCs, miR-1275 is controlled by a polycomb-mediated silencing mechanism and regulates expression of the oligodendroglial-lineage gene claudin 11 (*CLDN11*). These data illustrate that miR-1275 is regulated by an epigenetic pathway and that it contributes to the phenotypic diversity of glioblastoma tissues. The increased insight into the roles of these miRs may provide a better understanding of basis for the heterogeneity of glioblastomas in the context of human neurodevelopment (Katsushima et al., 2012). Recently, miR-204 was shown to suppress self-renewal, a stem cell characteristic, and the migration of GSCs by targeting the stemness-governing transcriptional factor *SOX4* and the migration-promoting receptor *EphB2* (Ying et al., 2013).

LncRNAs IN CANCER

Genome-wide studies showed that there are a large number of ncRNAs, including a group termed lncRNAs (Birney et al., 2007). LncRNAs are generally greater than 200 nucleotides and up to 100 kb in length (Mercer et al., 2009). It is known that lncRNAs are mainly transcribed by RNA polymerase II, are polyadenylated and spliced (Wu et al., 2008; Mercer et al., 2009; Ponting et al., 2009). Approximately 15,000 lncRNAs are estimated to occur in human cells and these are frequently expressed in tissue-specific patterns (Derrien et al., 2012). lncRNAs appear to play important roles in a wide range of biological cellular processes including maintenance of stemness, development, and cell survival (Koziol and Rinn, 2010; Zhang et al., 2013). Currently studies detected a set of lncRNAs in each disease using RNA immunoprecipitation with RNA binding proteins coupled with computational approaches.

Long non-coding RNAs are believed to regulate gene expression through four different pathways (Koziol and Rinn, 2010; Hu et al., 2012). First, lncRNAs can bind to chromatin modifying proteins (which have a scaffold function) and recruit these proteins to target loci. These lncRNA complexes can target genes that are closely situated in the genome (*cis*-regulation) or genes that are genomically distant (*trans*-regulation; Nagano et al., 2008; Pandey et al., 2008; Zhao et al., 2008; Gupta et al., 2010; Huarte et al., 2010; Tian et al., 2010; Prensner et al., 2011; Wang et al., 2011). Second, lncRNAs can act as an RNA decoy, that is, they can interact directly with a DNA binding domain to prevent transcription factors interacting with their DNA targets (Kino et al., 2010; Ng et al., 2012). Third, lncRNAs can act as an miRNA sponge, that is, they prevent specific miRNAs from binding to their target mRNAs by competitive binding (Poliseno et al., 2010; Cesana et al., 2011; Karreth et al., 2011). Fourth, lncRNAs can bind to specific combinations of regulatory proteins, such as RNA splicing proteins within ribonucleoprotein complexes (Tripathi et al., 2010; Ng et al., 2012; Schor et al., 2012).

There is increasing evidence to show that a set of lncRNAs is associated with cancer pathogenesis and that these lncRNAs function as regulators in cancer development (Prensner and Chinnaiyan, 2011). lncRNAs that are dysregulated in cancers are listed in **Table 2**. Below, we provide a brief description of some lncRNAs that are associated with glioma tumorigenesis.

Table 2 | List of lncRNAs dysregulated in cancers.

Name	Cancer type	Biological function	Molecular function	References
Oncogenic				
HOTAIR	Breast, hepatocellular, colorectal, pancreatic, GIST	Promotes invasion and metastasis, modulates cancer epigenome	Scaffold (PRC2, LSD1), guide (*trans*-regulation)	Gupta et al. (2010), Kogo et al. (2011), Yang et al. (2011), Niinuma et al. (2012), Kim et al. (2013)
ANRIL	Prostate, leukemia, melanoma	Suppresses senescence via INK4A	Scaffold (PRC1, PRC2), guide (*cis*-regulation)	Pasmant et al. (2007), Yu et al. (2008), Popov and Gil (2010), Pasmant et al. (2011)
MALAT1	Lung, prostate, breast, colon, hepatocellular	Regulates alternative splicing of pre-mRNA	Splicing (nuclear paraspeckle)	Ji et al. (2003), Muller-Tidow et al. (2004), Lin et al. (2007), Tano et al. (2010), Tripathi et al. (2010)
PCAT-1	Prostate	Promotes cell proliferation, inhibits BRCA2	Scaffold (PRC2), guide (*trans*-regulation)	Prensner et al. (2011)
CTBP1-AS	Prostate	Promotes cell proliferation	Scaffold (PSF), guide (*trans*-regulation)	Takayama et al. (2013)
PCGEM1	Prostate	Inhibits apoptosis, promotes cell proliferation	Unknown	Srikantan et al. (2000), Petrovics et al. (2004)
TUC338	Hepatocellular	Promotes cell proliferation	Unknown	Braconi et al. (2011)
uc. 73a	Leukemia, colorectal	Promotes cell proliferation, inhibits apoptosis	Unknown	Calin et al. (2007)
SPRY4-IT1	Melanoma	Promotes cell proliferation and invasion, inhibits apoptosis	Unknown	Khaitan et al. (2011)
ncRAN	Neuroblastoma, bladder	Promotes cell proliferation and invasion	Unknown	Yu et al. (2009), Zhu et al. (2011)
PRNCR1	Prostate	Promotes cell proliferation	Unknown	Chung et al. (2011)
H19	Breast, hepatocellular	Promotes cell proliferation, both oncogenic and tumor suppressive functions reported	Unknown	Gabory et al. (2006), Matouk et al. (2007)
Tumor suppressive				
GAS5	Breast	Induces growth arrest and apoptosis	Decoy (glucocorticoid receptor)	Mourtada-Maarabouni et al. (2008), Kino et al. (2010)
MEG3	Meningioma, hepatocellular, leukemia, pituitary, gliomas	Mediates p53 signaling, inhibits cell proliferation	Unknown	Zhou et al. (2007, 2012), Wang et al. (2012)
PTENP1	Prostate, colon	Inhibits cell proliferation	Sponge (PTEN)	Poliseno et al. (2010)
LincRNA-p21	Mouse models of lung, sarcoma, lymphoma	Induces apoptosis by repressing p53 targets	Scaffold (hnRNP-k), guide (*trans*-regulation)	Huarte et al. (2010)

MEG3

Maternally expressed gene 3 (*MEG3*) is a maternally expressed imprinted gene that can also act as an lncRNA. *MEG3* is generally expressed in normal tissues, and its downregulation by aberrant DNA methylation has been found in many types of human cancer (Zhou et al., 2012; Shi et al., 2013). For example, *MEG3* expression in glioma tissues is decreased compared to adjacent normal tissues (Wang et al., 2012). The tumor-suppressive role of *MEG3* is supported by the fact that it can associate with p53 and that this association is required for p53 activation (Lu et al., 2013). Ectopic expression of *MEG3* inhibits cell proliferation and induced cell apoptosis in glioma cell lines (Wang et al., 2012).

CRNDE

Colorectal neoplasia differentially expressed (*CRNDE*) transcripts are categorized as lncRNAs and have the potential to interact with chromatin-modifying proteins to regulate gene expression through epigenetic changes (Ellis et al., 2012). *CRNDE* is expressed in the fetal brain and in induced pluripotent stem cells; the level of expression increases during neuronal differentiation but no transcripts can be detected in the adult brain (Lin et al., 2011). Intriguingly, *CRNDE* is highly expressed in gliomas. The recent study of Ellis et al. demonstrated a direct interaction between *CRNDE* transcripts and components of PRC2 and the CoREST chromatin-modifying complex. *CRNDE* provides specific functional scaffolds for regulatory complexes, such as PRC2 and CoREST, and may contribute the maintenance of pluripotent state as well as neuronal differentiation (Ellis et al., 2012).

CONCLUDING REMARKS

Following the discovery of cancer stem cells, it became important to elucidate the mechanisms and the environmental cues that control the differentiation of these cells into the diverse array of cell types that form during tumorigenesis. Epigenetic dysregulation has recently been shown to change the balance between differentiation and self-renewal of cortical progenitor cells and, thereby, to alter the rate and developmental timing of neurogenesis (Pereira et al., 2010). Given that cancer is a disease of faulty cellular differentiation, it is likely that aberrant epigenetic mechanisms involving ncRNAs are involved in glioma tumorigenesis. lncRNAs are increasingly important because of their potential for use in clinical diagnosis and treatment. To date, however, the functions of only a few lncRNAs have been elucidated with respect to tumor biology and there are still many aspects that remain to be resolved. Further investigations are required to clarify the functional roles of lncRNAs in order to elucidate the gene regulatory mechanisms in gliomagenesis. Understanding of the interplays between lncRNAs and genomes, which are reversible alterations, may offer a novel opportunity for the development of molecularly targeted therapies. Nevertheless, a better understanding of the glioblastoma core signaling pathways regulated by ncRNAs and other epigenetic mechanisms will undoubtedly provide novel therapeutic targets and strategies with applications in diagnosis and therapy in glioblastoma.

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Aberrantly methylated genes in human papillary thyroid cancer and their association with *BRAF*/*RAS* mutation

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Cancer arises through accumulation of epigenetic and genetic alteration. Aberrant promoter methylation is a common epigenetic mechanism of gene silencing in cancer cells. We here performed genome-wide analysis of DNA methylation of promoter regions by Infinium HumanMethylation27 BeadChip, using 14 clinical papillary thyroid cancer samples and 10 normal thyroid samples. Among the 14 papillary cancer cases, 11 showed frequent aberrant methylation, but the other three cases showed no aberrant methylation at all. Distribution of the hypermethylation among cancer samples was non-random, which implied existence of a subset of preferentially methylated papillary thyroid cancer. Among 25 frequently methylated genes, methylation status of six genes (*HIST1H3J, POU4F2, SHOX2, PHKG2, TLX3, HOXA7*) was validated quantitatively by pyrosequencing. Epigenetic silencing of these genes in methylated papillary thyroid cancer cell lines was confirmed by gene re-expression following treatment with 5-aza-2′-deoxycytidine and trichostatin A, and detected by real-time RT-PCR. Methylation of these six genes was validated by analysis of additional 20 papillary thyroid cancer and 10 normal samples. Among the 34 cancer samples in total, 26 cancer samples with preferential methylation were significantly associated with mutation of *BRAF*/*RAS* oncogene ($P = 0.04$, Fisher's exact test). Thus, we identified new genes with frequent epigenetic hypermethylation in papillary thyroid cancer, two subsets of either preferentially methylated or hardly methylated papillary thyroid cancer, with a concomitant occurrence of oncogene mutation and gene methylation. These hypermethylated genes may constitute potential biomarkers for papillary thyroid cancer.

Keywords: DNA methylation, thyroid cancer, CIMP (CpG island methylator phenotype), BRAF, RAS, oncogene mutation

INTRODUCTION

Papillary thyroid cancer is the most common cancer derived from follicular cells. It is estimated that approximately 23,500 cases of differentiated thyroid cancer occur per year in the United States (Jemal et al., 2005), and 19,000 papillary thyroid cancer cases per year in the European Union (http://globocan.iarc.fr/). In Japan, about 8000 patients suffer from thyroid cancer every year, 80% of which are papillary cancer. While prognosis for papillary thyroid cancer is generally good, with a 10-year survival rate above 90%, some patients die of distant metastases and/or repeated recurrence (Ezaki et al., 1992; Yamashita et al., 1998).

RET/PTC (Rearranged in Transformation/Papillary Thyroid Carcinoma) re-arrangement, *BRAF* (V-Raf murine sarcoma viral oncogene homolog B) and *RAS* (Rat Sarcoma viral oncogene homolog) point mutations are frequently observed in papillary thyroid cancer (Mitsutake et al., 2006; Knauf and Fagin, 2009). Mutation of T to A at 1799 in the exon 15 of the *BRAF* gene has been reported in 28–69% of papillary thyroid cancer cases, while point mutations of *RAS* genes are detected in 5–20% (Cohen et al., 2003; Kimura et al., 2003; Namba et al., 2003; Kondo et al., 2007). Papillary thyroid cancer with poor prognosis is associated with *BRAF* mutation (Xing et al., 2005; Lee et al., 2012), whereas concomitantly, lengthy disease-free interval is not (Ulisse et al., 2012).

Patients with papillary thyroid cancer are generally treated by surgery. But it is difficult to decide whether total thyroidectomy, hemithyroidectomy, prophylactic central neck dissection or no dissection, should be performed in patients without preoperative or intraoperative evidence of metastatic lymph nodes (Xing et al., 2013). Association of *BRAF* mutation with occult central neck lymph node metastases (Joo et al., 2012) might support use of *BRAF* mutation as an indication for prophylactic central neck dissection for patients with conventionally low- to intermediate-risk papillary thyroid cancer. But precise diagnosis to define prognosis and suitable therapy is currently impossible. Molecular biomarkers would therefore simplify disease management (McLeod et al., 2013).

Along with genetic alterations, accumulation of epigenetic alterations is known to affect cancer development (Baylin and Ohm, 2006; Feinberg et al., 2006; Esteller, 2007). Aberrant DNA methylation at promoter regions is a major epigenetic alteration to silence tumor suppressor genes in many cancer types. *RASSF1A* (Ras association (RalGDS/AF-6) domain family member 1) is methylated in 20% of papillary thyroid cancer, leading to activation of the RAS-MAPK (Mitogen-Activated Protein Kinase) signal (Xing et al., 2004). Papillary thyroid cancer is also reported to involve methylation of other genes, including *RARB* (Retinoic Acid Receptor, Beta), $p16^{INK4A}$ (*CDKN2A*, Cyclin-Dependent Kinase Inhibitor 2A), *TSHR* (Thyroid Stimulating Hormone Receptor), *CDH1* (Cadherin 1, type 1, E-cadherin), *DAPK* (Death-Associated Protein Kinase 1), and *MLH1* (mutL Homolog 1) (Hoque et al., 2005; Guan et al., 2008; Mohammadi-asl et al., 2011). While a few genes known to be aberrantly methylated in other cancers were analyzed in these studies, methylation frequencies ranged from 15 to 33%. Involvement of genes in aberrant DNA methylation, however, has not been well-clarified in papillary thyroid cancer. Whether any subset of papillary thyroid cancer shows preferential aberrant methylation, and whether such methylation and other clinicopathological factors are associated are also unclear.

We here analyzed DNA methylation status of promoter regions on a genome-wide scale, using the Illumina Infinium HumanMethylation27 BeadChip technique on 14 clinical papillary thyroid cancer samples and 10 normal thyroid samples. For genes frequently hypermethylated in cancer, methylation status was validated quantitatively by pyrosequencing, using 20 additional clinical cancer samples and 10 additional normal samples. Methylation-associated gene silencing was confirmed by gene re-expression following 5-aza-2′-deoxycytidine and trichostatin A treatment, and by quantitative reverse transcription-polymerase chain reaction (RT-PCR) on thyroid cancer cell lines. We found a number of genes with frequent aberrant methylation and silencing in papillary thyroid cancer, and a subset of cancer with preferential aberrant methylation.

MATERIALS AND METHODS

CLINICAL SAMPLES AND CELL LINES

We obtained 79 primary papillary thyroid cancer samples from patients who underwent thyroidectomy at The University of Tokyo, with written informed consent. These samples were immediately frozen with liquid nitrogen and stored at −80°C. The frozen materials were microscopically examined for cancer cell contents by pathologists and were dissected to enrich cancer cells when necessary. Thirty-four cancer samples containing more than 40% of cancer cells were selected and used for further analysis. DNA was extracted by QIAamp DNA Micro Kit (QIAGEN, Valencia, CA). Thyroid cancer cell line BHT-101 was obtained from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures), TPC-1 was provided from Dr. Mitsutake, University of Nagasaki (Ishizaka et al., 1989) and KTC-1 and KTC-3 cell lines were provided from Dr. Kurebayashi, Kawasaki Medical University (Kurebayashi et al., 2000, 2006). These cell lines were maintained in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, streptomycin sulfate (100 mg/L), and penicillin G sodium (100 mg/L). Peripheral blood cell samples were purchased from The Coriell Cell Repositories. This research was certified by the Ethics Committee of The University of Tokyo and Chiba University.

INFINIUM ASSAYS

High-resolution methylation analysis was conducted on the Illumina Infinium HumanMethylation27 microarray platform. This BeadChip assay measures methylation, given as a β-value, at more than 27,000 CpG loci covering 15,000 genes. For each CpG site, the β-value is the ratio of the fluorescence signal from the methylated probe over the sum of methylated and unmethylated probe signals. The β-value, ranging from 0.00 to 1.00, reflects the methylation level of the individual CpG site. Bisulfite conversion, whole-genome amplification, labeling, hybridization, and scanning were carried out according to the manufacturer's protocols. According to the previously proposed classification (Weber et al., 2007), Infinium probes were classified into three categories: high-CpG, intermediate-CpG, and low-CpG probes, on the basis of CpG ratio (the ratio of observed CpG count over expected CpG count) and GC contents within 500 bp region around the probe site (Matsusaka et al., 2011). Genes in X and Y chromosomes were excluded to avoid gender differences. Infinium enables us to analyze DNA methylation levels systematically for more than 14,000 genes, but methylation level of a single CpG site detected by Infinium may not precisely represent methylation status of promoter CpG island. Some Infinium probes might be less quantitative; in analysis of control samples with known levels of methylation (0, 25, 50, 75, 100%), the observed β-values generally correlated with the expected β-values, while some probes showed lower β-values (0.0–0.3) for 75% control or higher β-values (0.7–1.0) for 25% control (Nagae et al., 2011).

PYROSEQUENCING ANALYSIS

Quantitative validation for methylation status was carried out by pyrosequencing as previously reported (Matsusaka et al., 2011). Primers were designed to include no or only one CpG site in a primer sequence using Pyro Q-CpG Software (QIAGEN), to amplify bisulfite-treated DNA regions containing several CpG sites. For C of CpG site within a primer sequence, a nucleotide which does not anneal to C or U was chosen, e.g., adenosine (A). Briefly, the biotinylated PCR product was bound to streptavidin sepharose beads HP (Amersham Biosciences, Sweden), washed, and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified, single-stranded PCR product, pyrosequencing was carried out using PyroMark Q96 ID System (QIAGEN) according to the manufacturer's instructions. Primer sequences and conditions for *HIST1H3J* (Histone cluster 1, H3j), *POU4F2* (POU class 4 homeobox 2), *SHOX2* (Short stature homeobox 2), *PHKG2* (Phosphorylase Kinase, Gamma 2), *TLX3* (T-cell Leukemia homeobox 3), and *HOXA7* (Homeobox A7), are shown in **Table 1**. Control samples with known levels of methylation (0, 25, 50, 75, 100%) were prepared as previously described (Yagi et al., 2010). Pyrosequencing is not systematic, but highly quantitative (Matsusaka et al., 2011), and enables us to precisely validate the methylation level at one

Table 1 | Primer sequences for pyrosequencing of six potential methylation biomarkers.

Genes	Primer sequences for PCR (Forward/Reverse)		Sequencing primers
HIST1H3J	F	GTTATAAATTTTGGTAGAAGTTATTGT	ATGGTTAGGAAGAAGTAGATAGT
	R*	ACCTTAATAACCAACTACTTCC	
POU4F2	F	GGGGAGAGGGGAGTATAA	ATTAGTTTAGATTGATAGTAGAGG
	R*	AAAAAAAACTATACCAAATTAAACTCACCC	
SHOX2	F*	TTGGGGGGGTTGGAGTAGTAAA	AACCCCCTAAATTCTTCCAT
	R	CTCCTTCTTCTCCTTCACTTTCTAATC	
PHKG2	F	GTTTGTAATTTTAGTATTTTGGGAGGTTGA	AAGTTTAAGGTTGTAGTGA
	R*	TCCCTAACTAAATTCAACATTTTCTCTT	
TLX3	F	TGGTTGAGGTAGGAGAGGAATTAGTA	GGTTTAAGAAAGATGATATAGAG
	R*	CACTAAAACTTTACCAAAAATAC	
HOXA7	F*	GGGAGTAAAGGAGTAAGAAGT	CAACAAATCACAAATCAAAATTA
	R	ACCCAACAACAAATCACAAATCAAAATT	

**Primers with 5′-biotin tag.*

CpG site, as determined by the Infinium assay, as well as at surrounding CpG sites. Mean methylation levels of these CpG sites were calculated to represent methylation status of each gene promoter, and were displayed in figures by heatmap or dot chart.

Mutation analysis

Mutations at *BRAF* 1799, *KRAS* (Kirsten Rat Sarcoma viral oncogene homolog) 34, 35, 38, *NRAS* (Neuroblastoma RAS viral oncogene homolog) 181, 182, 183, *HRAS* (Harvey Rat Sarcoma viral oncogene homolog) 35, 37, 181, 182, 183, were analyzed by genotyping assays on MassARRAY platform, by detecting mass difference of the extended nucleotide using matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) (Yagi et al., 2012). First, PCR amplification primers and a post-PCR extension primer were designed using MassARRAY Assay Design 3.0 software (Sequenom), and listed in **Table 2**. Those mutations were analyzed in a single reaction by multiplex PCR. PCR amplification was performed in 5-μL volume containing 0.5 unit of Taq polymerase, 5 ng of genomic DNA, 0.5 pmol of PCR primer and 2.5 nmol of dNTPs. PCR reactions were cycled at 94°C for 15 min, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 1 min. Shrimp alkaline phosphatase treatment was performed at 37°C for 20 min and 85°C for 5 min. Post-PCR primer extension was carried out using 5.6 pmol of extension primer. Extension reaction was cycled at 94°C for 30 s, followed by 40 cycles of 94°C for 5 s, 5 cycles of 52°C for 5 s and 80°C for 5 s, and 72°C for 3 min. Reaction products were transferred to a SpectroCHIP (Sequenom) and mass difference was analyzed using MALDI-TOF-MS. Irradiation of the matrix-oligonucleotide-cocrystal with a laser beam ultimately results in desorption and ionization of the oligonucleotides, which then can be accelerated in an electrical field into the TOF device. The TOF device separates the accelerated analyte ions of different mass-to-charge (m/z) ratios by providing a field-free drift tube of defined length. After passing through the tube, ions are detected; every signal is assigned to a specific molecular mass calculated from the TOF. The extended bases at possible mutation sites are determined from the difference of nucleotide molecular masses (Vogel et al., 2009).

5-Aza-2′-DEOXYCYTIDINE AND TRICHOSTATIN A TREATMENT

Thyroid cancer cell lines were cultured at a density of 3×10^5 cells/10-cm dish on Day 0. Cells were exposed to 3 μM 5-aza-2′-deoxycytidine (Sigma-Aldrich, St. Louis, MO) on Days 1, 2, and 3, and to 300 nM trichostatin A (Sigma-Aldrich) on the Day 3. 5-Aza-2′-deoxycytidine induces hypomethylation of DNA by inhibiting DNA methyltransferase, and re-expression of silenced genes by 5-aza-2′-deoxycytidine treatment is synergistically enhanced by trichostatin A, a histone deacetylase inhibitor (Suzuki et al., 2002). 5-Aza-2′-deoxycytidine is unstable in aqueous solution, and thus a 20 mM solution in dimethyl sulfoxide (DMSO) was freshly prepared, and diluted in medium to 3 μM every day immediately before medium change. The medium was changed every 24 h, and the cells were harvested on Day 4.

QUANTITATIVE PCR ANALYSIS

RT-PCR was performed using CFX96 Touch TM Real-Time PCR Detection System (BIORAD Laboratories). cDNA was synthesized from 1 μg of total RNA treated with DNase I with a Superscript III kit (Invitrogen, Life Technologies). The quantity of cDNA of each gene in a sample was measured by comparing it with standard samples that contained 10^1 to 10^6 copies of the genes, and normalized to that of *PPIA* (Peptidylprolyl Isomerase A). Primer sequences are shown in **Table 3**.

STATISTICAL ANALYSIS

P-values were calculated to compare methylation(+) cancer and methylation(−) cancer and to analyze the correlation of the methylation status to clinicopathological features. Fisher's exact test was used for analysis of binary features such as sex, distant metastasis, recurrence, and mutation of *BRAF*/*RAS* oncogenes (with simple choice between male and female, occurrence and no occurrence); *t*-test was used for more descriptive features that do not imply a choice, such as age, tumor size, number of lymph nodes with metastasis, and thyroglobulin. When $P < 0.05$, the correlation was considered statistically significant. *P*-values were also calculated by *t*-test to compare distribution of methylation

Table 2 | Primer sequences used for mutation analysis (MALDI-TOF-MS assays).

Mutation sites	Primer sequences (Forward/Reverse)		Extend primers
*BRAF*_1799	F	ACGTTGGATGTTCAAACTGATGGGACCCAC	TGATTTTGGTCTAGCTACAG
	R	ACGTTGGATGTCTTCATGAAGACCTCACAG	
*HRAS*_34	F	ACGTTGGATGAATGGTTCTGGATCAGCTGG	ACTCTTGCCCACACCGC
	R	ACGTTGGATGGACGGAATATAAGCTGGTGG	
*HRAS*_35	F	ACGTTGGATGAATGGTTCTGGATCAGCTGG	AGCGGGTGGTGGTGGGCGCCG
	R	ACGTTGGATGGACGGAATATAAGCTGGTGG	
*HRAS*_37	F	ACGTTGGATGAATGGTTCTGGATCAGCTGG	TCATCGCACTCTTGCCCACAC
	R	ACGTTGGATGGACGGAATATAAGCTGGTGG	
*HRAS*_38	F	ACGTTGGATGAATGGTTCTGGATCAGCTGG	CAGCGCACTCTTGCCCACA
	R	ACGTTGGATGGACGGAATATAAGCTGGTGG	
*HRAS*_181	F	ACGTTGGATGTGGCAAACACACACAGGAAG	CATGGCGCTGTACTCCTCCT
	R	ACGTTGGATGTGTTGGACATCCTGGATACC	
*HRAS*_182	F	ACGTTGGATGTGGCAAACACACACAGGAAG	CATGGCGCTGTACTCCTCC
	R	ACGTTGGATGTGTTGGACATCCTGGATACC	
*HRAS*_183	F	ACGTTGGATGTGGCAAACACACACAGGAAG	CGCATGGCGCTGTACTCCTC
	R	ACGTTGGATGTGTTGGACATCCTGGATACC	
*KRAS*_34	F	ACGTTGGATGTAGCTGTATCGTCAAGGCAC	ACTCTTGCCTACGCCAC
	R	ACGTTGGATGAGGCCTGCTGAAAATGACTG	
*KRAS*_35	F	ACGTTGGATGTAGCTGTATCGTCAAGGCAC	CTGTGGTAGTTGGAGCTG
	R	ACGTTGGATGAGGCCTGCTGAAAATGACTG	
*KRAS*_37	F	ACGTTGGATGTAGCTGTATCGTCAAGGCAC	GAGGGGCACTCTTGCCTACGC
	R	ACGTTGGATGAGGCCTGCTGAAAATGACTG	
*KRAS*_38	F	ACGTTGGATGTAGCTGTATCGTCAAGGCAC	AGGCACTCTTGCCTACG
	R	ACGTTGGATGAGGCCTGCTGAAAATGACTG	
*NRAS*_181	F	ACGTTGGATGTCGCCTGTCCTCATGTATTG	ATACTGGATACAGCTGGA
	R	ACGTTGGATGCCTGTTTGTTGGACATACTG	
*NRAS*_182	F	ACGTTGGATGTCGCCTGTCCTCATGTATTG	ATGGCACTGTACTCTTCT
	R	ACGTTGGATGCCTGTTTGTTGGACATACTG	
*NRAS*_183	F	ACGTTGGATGTCGCCTGTCCTCATGTATTG	CTGGATACAGCTGGACA
	R	ACGTTGGATGCCTGTTTGTTGGACATACTG	

Table 3 | Primer sequences for real-time RT-PCR in gene re-expression analysis.

Gene	Primer sequences (Forward/Reverse)		Anneal (°C)	Product (bp)
HIST1H3J	F	AAATCAAGCAGAGGCGAAGTCGGA	58	106
	R	GGATAGTGGGTCTCGTCAAAAAGC		
POU4F2	F	CACCAAGCCTGAACTCTTCAAT	58	101
	R	GCTGAATGGCAAAGTAGGCTTCG		
SHOX2	F	AAATCAAGCAGAGGCGAAGTCGGA	58	85
	R	GGATAGTGGGTCTCGTCAAAAAGC		
PHKG2	F	TGATCTTGTTCACACTCCTGGCT	58	145
	R	GAGATCAGGTCTTTGACAGTGCT		
TLX3	F	CTGTCTGCACAACTCGTCACTCTT	60	79
	R	GACAGCGGGAACCTTGGAACTATC		
HOXA7	F	AGTTCCACTTCAACCGCTACCTGAC	58	131
	R	GTCCTTATGCTCTTTCTTCCACTTC		

ratios between cancer and normal samples. When $P < 0.05$, the difference of the methylation ratios between cancer and normal samples was considered statistically significant. The dot chart and heatmap were drawn using Excel software and Java TreeView software (http://jtreeview.sourceforge.net/).

RESULTS

ONCOGENE MUTATION STATUS IN PAPILLARY THYROID CANCER

We analyzed mutation status of *BRAF* and *RAS* (*HRAS*, *NRAS*, and *KRAS*) oncogenes in 34 papillary thyroid cancer samples using MALDI-TOF-MS (**Figure 1**). *BRAF* mutation was detected

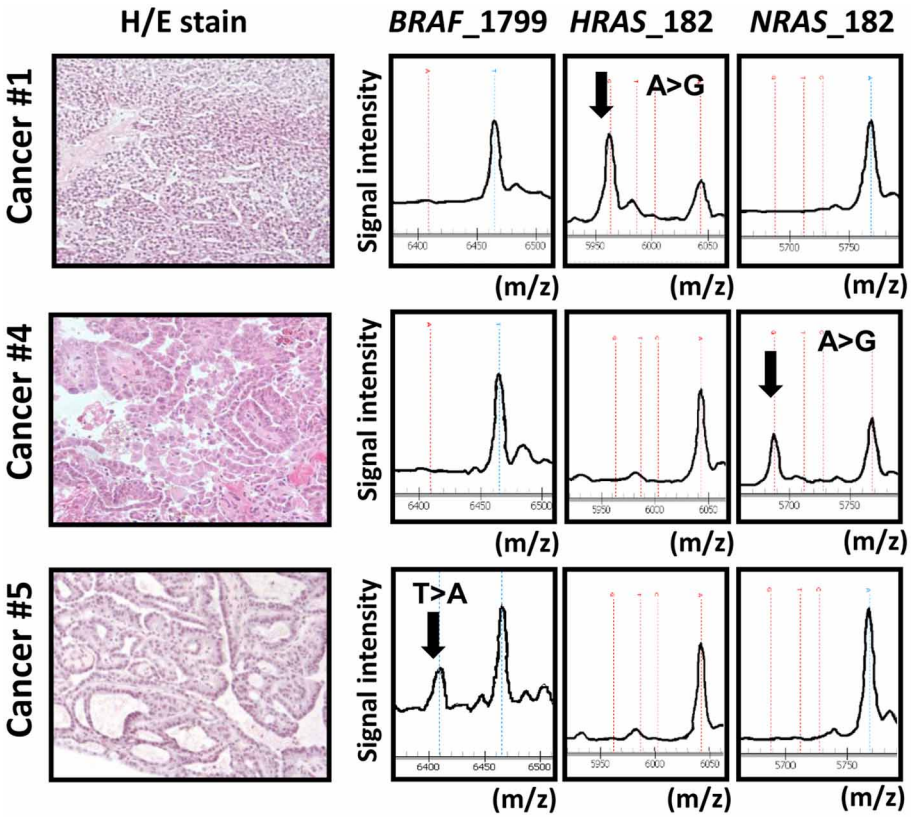

FIGURE 1 | Representative data obtained from MALDI-TOF-MS imaging for three cancer samples. Cancer samples #1, #4, and #5 correspond to papillary cancer samples #1, #4, and #5 in **Figure 3**. First column: photomicrograph of a tumor section with hematoxylin/eosin (H/E) staining. Second, third, and fourth columns: MALDI-TOF-MS profile with detection of *BRAF*/*RAS* mutations. *X*-axis indicates mass-to-charge ratio (m/z) to distinguish wild-type and mutant allele, and Y-axis indicates signal intensity.

in 67% (23/34) of the 34 samples, whereas *HRAS*, *NRAS*, and *KRAS* mutations were detected less frequently, in 3% (1/34), 3% (1/34), and 0% (0/34) sample, respectively. Each oncogene mutation was mutually exclusive; 25 among the 34 samples (75%) were oncogene-mutation(+) cancer.

DNA METHYLATION ANALYSIS USING ILLUMINA INFINIUM BEADARRAY

Among 34 papillary thyroid cancer and 24 normal thyroid samples, 14 and 10 samples, respectively, were analyzed using Infinium 27K BeadArray. Methylation data of other cancer types (80 head and neck squamous cell cancers, 50 gastric cancers, 80 colorectal cancers, 80 prostate cancers, 24 chronic myeloid leukemias, 50 glioblastomas), and normal samples of corresponding tissues were collected from National Center for Biotechnology Information, Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/gds: GSE25089 for head and neck squamous cell carcinoma, GSE31789 for gastric cancer, GSE27130 for colorectal cancer, GSE26126 for prostate cancer, GSE31600 for chronic myeloid leukemia, and GSE22867 for glioblastoma). To analyze aberrantly methylated genes in cancer samples, probes with β-value < 0.17 in all the normal samples and with standard deviation of β-values in cancer samples >0.15 were selected, and shown in **Figure 2**. Each cancer type including papillary thyroid cancer showed a unique pattern of aberrant promoter methylation.

ABERRANTLY METHYLATED GENES IN PAPILLARY THYROID CANCER

While the number of aberrantly methylated genes was relatively small in papillary thyroid cancer (**Figure 2**), 25 genes showed frequent hypermethylation ($\beta > 0.25$) in three or more samples among the 14 papillary thyroid cancer samples, and no methylation ($\beta < 0.2$) in all the 10 normal samples (**Figure 3**). To check that the hypermethylation status was not due to contaminated blood cells, the methylation status of these genes in peripheral blood cells was also analyzed to see that none of them were methylated in blood (**Figure 3**).

Among 14 papillary cancer samples, 11 samples showed aberrant methylation in three or more genes, whereas the other three samples showed no aberrant methylation at all (**Figure 3**). When methylation status was compared with clinicopathological factors, the two cancer cases with recurrence were both methylation-negative ($P = 0.03$, Fisher's exact test) (**Figure 3**). Nine of the 11 frequently methylated samples showed mutation of *BRAF*/*RAS* oncogenes, whereas none of the three methylation-negative samples showed oncogene mutation ($P = 0.03$, Fisher's exact test). Other clinicopathological factors, including tumor size, lymph

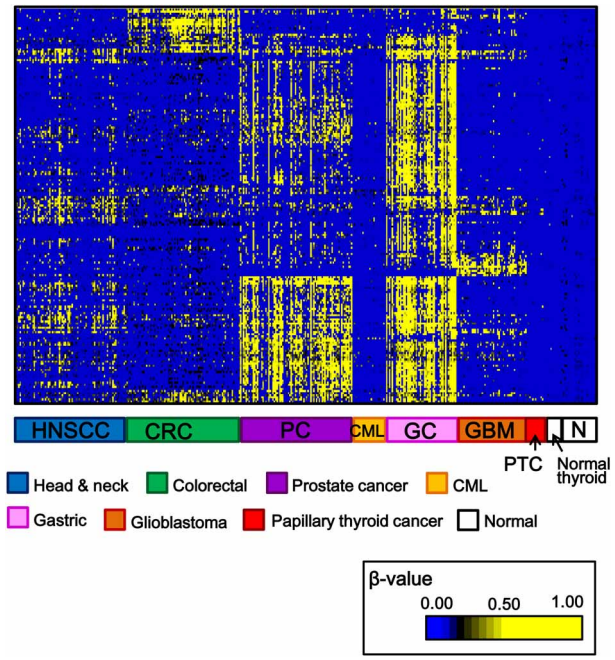

FIGURE 2 | Heatmap for methylation status of several cancer types. Genes with β-value <0.17 in normal samples and with standard deviation of β-value in cancer samples >0.15, were selected and their β-values were shown by heatmap. Each cancer type showed different methylation profile, and papillary thyroid cancer displayed fewer aberrantly methylated genes than other cancer types. HNSCC, head and neck squamous cell carcinoma; CRC, colorectal cancer; PC, prostate cancer; CML, chronic myeloid leukemia; GC, gastric cancer; GBM, glioblastoma; PTC, papillary thyroid cancer; N, normal samples corresponding to these cancer types.

node metastasis, distant metastasis, tumor stage, age, or sex, did not show significant difference.

To validate the methylation status of these genes, six out of the 25 genes, *HIST1H3J*, *POU4F2*, *SHOX2*, *PHKG2*, *TLX3*, and *HOXA7*, were randomly chosen and analyzed by pyrosequencing, a highly quantitative method (**Figure 4**). Although one normal sample showed high methylation in *POU4F2*, frequent hypermethylation of these genes in papillary cancer samples was confirmed, while normal thyroid samples were generally unmethylated.

EVALUATION OF GENE SILENCING

The analyzed tissue samples include a part of non-tumor cells (see Materials and Methods). To evaluate whether these aberrantly methylated genes were silenced in cancer cells, we analyzed methylation status of these six genes in papillary thyroid cancer cell lines (TPC1, KTC1, and KTC3) and anaplastic thyroid cancer cell line BHT-101 (**Figure 5A**). All the genes except *SHOX2* showed dense methylation in at least one papillary thyroid cancer cell line, confirming that hypermethylation detected in cancer tissue samples is due to hypermethylation in cancer cells.

We next performed real-time RT-PCR for the six genes. All the genes except *SHOX2* showed no or very low expression in the analyzed, methylated cancer cell line, and showed re-expression in cells treated with 5-aza-2′-deoxycytidine and/or trichostatin A (**Figure 5B**). *SHOX2* was neither expressed, nor methylated in KTC1 (**Figure 5A**). Consequently, its expression was not reversed by the deoxycytidine/trichostatin treatment (**Figure 5B**). This is presumably because *SHOX2* was silenced in KTC1 by mechanisms other than promoter methylation, e.g., by depletion of appropriate transcription factors.

METHYLATION ANALYSIS OF THE SIX GENES IN ADDITIONAL SAMPLES

To statistically extend the validation of aberrant methylation of the six genes, we analyzed the methylation status in 20 additional papillary thyroid cancer samples and 10 additional normal thyroid samples by pyrosequencing (**Figure 6**). A similar fraction of cancer samples showed high methylation in each gene (2/20 for *HIST1H3J*, 8/20 for *POU4F2*, 4/20 for *SHOX2*, 5/20 for *PHKG2*, 6/20 for *TLX3*, and 4/20 for *HOXA7*).

When methylation ratios were compared between 34 cancer samples in total and 20 normal samples in total, five genes (*HIST1H3J*, *SHOX2*, *PHKG2*, *TLX3*, and *HOXA7*) showed significantly higher methylation in cancer ($P < 0.05$, ranging from 0.0001 to 0.004, t-test), and *POU4F2* tended to show higher methylation in cancer ($P = 0.07$, t-test) (**Figure 7A**). Among the 34 cancer samples, 26 showed aberrant methylation in at least one gene, but eight showed no aberrant methylation at all (**Figure 7B**). When clinicopathological features were compared between methylation(+) cancer and methylation(−) cancer, mutations of *BRAF*/*RAS* oncogenes significantly correlated to methylation(+) groups ($P = 0.04$, Fisher's exact test) (**Table 4**). Although it was not statistically significant, methylation(+) cancer tended to show larger size of tumor ($P = 0.06$, t-test) and higher levels of thyroglobulin ($P = 0.08$, t-test).

DISCUSSION

In this study, we performed genome-wide DNA methylation analysis in 14 human papillary thyroid cancer samples and 10 normal samples. Although papillary thyroid cancer apparently involves fewer aberrantly methylated genes than other types of cancers, we detected 25 genes frequently hypermethylated in papillary thyroid cancer. Methylation status was quantitatively validated in six out of the 25 genes by pyrosequencing, using the genome-widely analyzed samples and additional samples. Gene silencing in papillary thyroid cancer cell lines was confirmed by real-time RT-PCR. While a subset of cancer cases had no aberrant methylation at all, cancer with preferential methylation tended to have oncogene mutation and to be larger tumor.

Papillary thyroid cancer displayed fewer aberrantly methylated genes, compared with other cancer types (**Figure 2**). For genes previously reported to be methylated in thyroid cancer, such as *TSHR* (Xing et al., 2003), or in other cancer types, such as *RASSF1A*, *RAR*-β*2*, *p16*, *CDH1*, *DAPK*, and *MLH1*, the methylation frequency in papillary thyroid cancer ranges from 15 to 33% (Hoque et al., 2005; Guan et al., 2008; Mohammadi-asl et al., 2011). In these reports, no or few normal samples were analyzed (Guan et al., 2008; Mohammadi-asl et al., 2011), methylation was also detected in normal samples (Hoque et al., 2005), or a non-quantitative method, i.e., standard methylation-specific

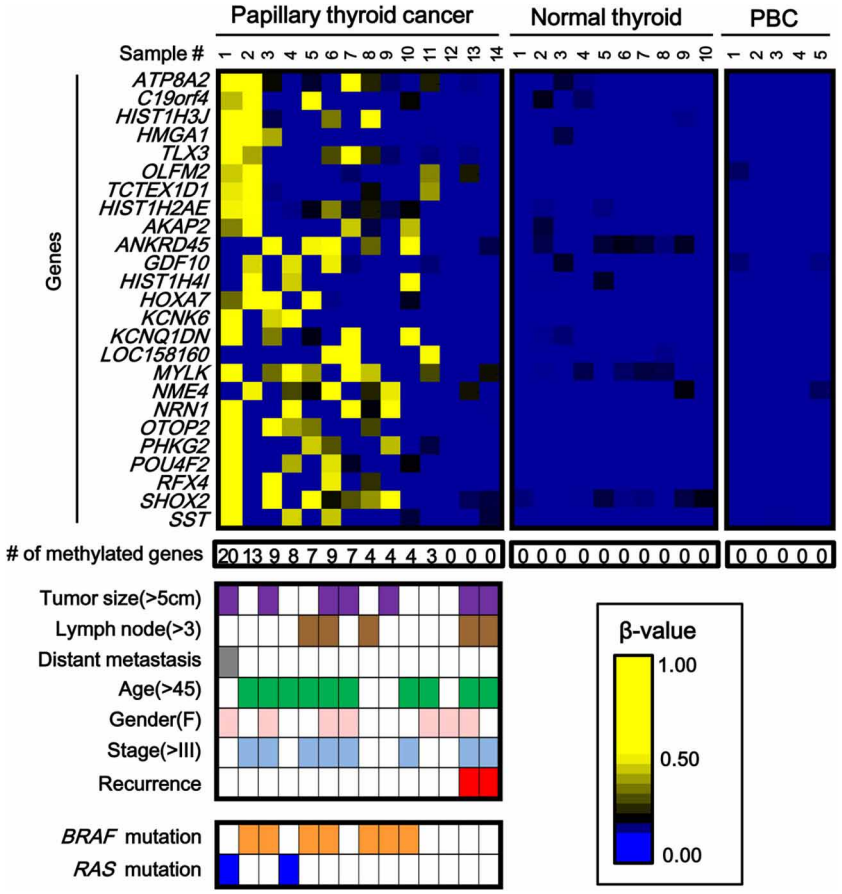

FIGURE 3 | Aberrantly methylated genes frequently observed in papillary thyroid cancer. PBC, peripheral blood cells. We detected 25 genes that were hypermethylated ($\beta > 0.25$) in at least three of 14 papillary thyroid cancer samples, but not methylated ($\beta < 0.2$) in 10 normal samples. Among the 14 papillary cancer samples, 11 showed aberrant methylation in three or more genes, whereas the other three samples had no aberrant methylation. Purple, tumor size; brown, number of metastatic lymph nodes >3; gray, distant metastasis(+); green, age over 45 years old; pink, female; pale blue, stage III or IV; red, recurrence (+); orange, *BRAF* mutation(+); blue, *RAS* mutation (+). The two samples with recurrence (red) had no aberrant methylation ($P = 0.03$, Fisher's exact test). *BRAF*/*RAS* oncogene mutations were all observed in methylation(+) samples ($P = 0.03$, Fisher's exact test).

PCR, was used (Guan et al., 2008). Standard methylation-specific PCR (Herman et al., 1996) can amplify and detect minor fraction of methylated alleles, but its high sensitivity can lead to overestimation of methylation frequency. Our analysis did not select these genes as frequently methylated ones in the first 14 cancer samples, because normal thyroid tissues also showed high methylation levels or because methylation frequencies in papillary thyroid cancer samples were low ($\leq$2 of 14 cancer samples). Instead, we detected 25 novel genes that were frequently aberrantly methylated ($\beta > 0.25$) in at least three of the 14 cancer samples, and not methylated in any of the 10 normal thyroid samples ($\beta < 0.2$).

Interestingly, three of the 14 papillary thyroid cancer samples showed no aberrant methylation in the 25 genes, but the other 11 cancer samples showed hypermethylation in at least three of the 25 genes. No cancer sample showed aberrant methylation in just one or two genes. This unusual distribution of aberrant methylation is similar to the CpG island methylator phenotype (CIMP), which was first proposed in colorectal cancer (Toyota et al., 1999). As Toyota et al. demonstrated in colorectal cancer (Toyota et al., 1999), we calculated probability of methylation distribution in papillary thyroid cancer using these 25 genes. The fraction of methylated tumors in each gene was 3/14 for *ATP8A2*, 3/14 for *C19orf4*, ..., 5/14 for *ANKRD45*, ..., 6/14 for *MYLK*, ..., 4/14 for *NRN1*, ..., 5/14 for *SHOX2*, and 3/14 for *SST* (**Figure 3**). Assuming that methylation of these genes is random, the probability that none of the 25 genes would be methylated in three cancer samples is $P = 1.2 \times 10^{-8}$. This was calculated using the following formula:

$$p(x) = \frac{\binom{N}{x}\prod_g \binom{N-x}{f(g)}}{\prod_g \binom{N}{f(g)}}$$

where x indicates number of samples which do not have methylated genes ($x = 3$ in the present case), N indicates number of cancer samples ($N = 14$ here), g indicates one of the 25 genes,

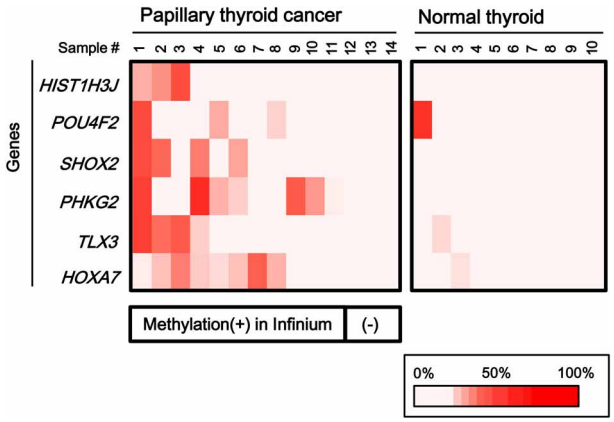

FIGURE 4 | Confirmation of aberrant methylation by pyrosequencing. Six genes were randomly chosen among the 25 frequently methylated genes, and their methylation status was quantitatively validated by pyrosequencing, using the 14 papillary thyroid cancer samples and 10 normal thyroid samples that were analyzed by Infinium. Although one normal sample showed high methylation in *POU4F2*, aberrant methylation of the six genes in the papillary cancer samples was confirmed. **Bottom**, the corresponding data for aberrant methylation obtained by Infinium analysis (**Figure 3**).

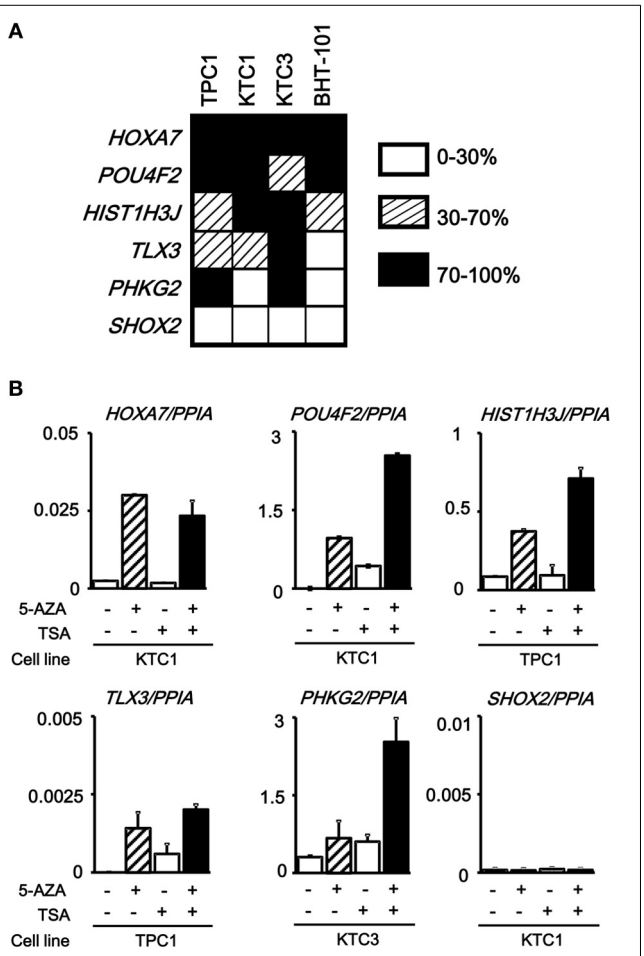

FIGURE 5 | Silencing of aberrantly methylated genes. (A) Methylation status of the *HOXA7, POU4F2, HIST1H3J, TLX3, PHKG2, SHOX2* genes was analyzed in papillary thyroid cancer cell lines TPC1, KTC1, and KTC3 and anaplastic thyroid cancer cell line BHT-101 by pyrosequencing. *Open box*, 0–30% methylation, presumably no allele methylation. *Hatched box*, 30–70% methylation, presumably hemi-allelic or partial methylation. *Closed box*, 70–100% methylation, presumably bi-allelic and dense methylation. All the genes except *SHOX2* showed dense methylation in at least one papillary thyroid cancer cell line. **(B)** Real-time RT-PCR analysis of the methylated genes. Cells were treated with 5-aza-2′-deoxycytidine (5-AZA) and/or trichostatin A (TSA). Gene expression levels were normalized to that of *PPIA* (*Peptidylprolyl Isomerase A*, or *cyclophilin A*). All the genes except *SHOX2* showed no or very low expression in the analyzed cancer cell line, and showed re-expression in cells treated with 5-AZA/TSA.

and $f(g)$ indicates the number of samples in which g is methylated. Similarly, the probability that at least three of the 25 genes are methylated in 11 cancer samples would be $P = 0.0028$. In each case, a random event is highly unlikely. We rather observe that the associated methylation or un-methylation events both occur at relatively high frequencies. Our data thus suggest that there are two distinct classes of papillary thyroid cancer. One is a subset of hardly methylated cancer. The second one is a subset of preferentially methylated cancer, prone to transcriptional silencing and with the potential to inactivate several genes simultaneously, as CIMP has been proposed in colorectal cancer and other cancers (Toyota et al., 1999; Kaneda et al., 2002; Noushmehr et al., 2010).

Although the number of analyzed samples was not large, preferentially methylated papillary thyroid cancer showed mutation of *BRAF*/*RAS* oncogenes more frequently than methylation(−) cancer ($P = 0.04$, Fisher's exact test). In previous studies of papillary thyroid cancer, although methylation of *RASSF1A* and *BRAF* mutation were detected in a mutually exclusive manner (Xing et al., 2004; Hoque et al., 2005), methylation of *RARβ2* or *MLH1* significantly correlated to *BRAF* mutation (Hoque et al., 2005; Guan et al., 2008). Correlation of aberrant methylation and oncogene mutation are also reported in colorectal cancer; high-methylation and intermediate-methylation epigenotypes strongly correlated to *BRAF* mutation and *KRAS* mutation, respectively, and low-methylation epigenotype strongly correlated to lack of oncogene mutation (Shen et al., 2007; Yagi et al., 2010; Hinoue et al., 2012). The mechanism of these correlations is still unknown, but oncogene mutation may somehow induce aberrant methylation, or aberrant methylation may help escape from senescence by disrupting factors critical in *RAF*/*RAS*-induced senescence (Kaneda and Yagi, 2011).

Preferentially methylated cancer also tended to have larger tumors and higher thyroglobulin levels, which might relate to cancer progression (Piccardo et al., 2013). Although 90% of papillary thyroid cancers are considered to be at low risk with a mortality rate of 1–2%, the mortality rate of the high risk group is 50–75% (Hay et al., 1993; Noguchi et al., 1994; Shaha et al., 1996; Dean and Hay, 2000). The tumor-node-metastasis (TNM) classification is a tool for cancer prognosis; each variable used in TNM staging (age, tumor size, extent of primary tumor, and presence of nodal or distant metastases) shows significant association with observed end points of cancer recurrence or death. Cancer

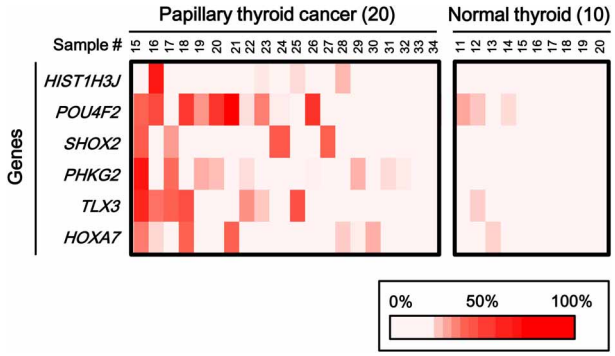

FIGURE 6 | Aberrant methylation of *HOXA7, POU4F2, HIST1H3J, TLX3, PHKG2, SHOX2* genes in additional samples: 20 cancer samples and 10 normal ones. Methylation status of the six genes was confirmed by pyrosequencing. Bottom, color scale with known methylation degrees (0–100%).

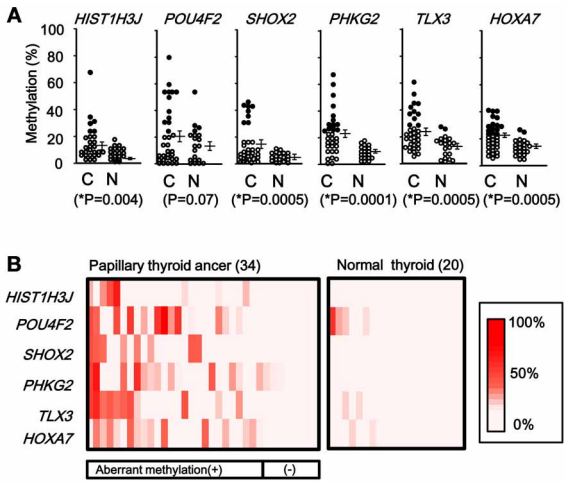

FIGURE 7 | Methylation status of the six genes in 54 samples analyzed by pyrosequencing. (A) Each gene was methylated to a different extent in each sample. *C*, 34 cancer samples. *N*, 20 normal samples. *Circle*, methylation ratio of each sample. Filled circles indicate that the gene is methylated with the methylation ratio >25%. *Bars*, the mean and standard error of methylation ratios. *P*-values were calculated by *t*-test to compare distribution of methylation ratios between cancer (*C*) and normal (*N*) samples. Five genes (*HIST1H3J, SHOX2, PHKG2, TLX3*, and *HOXA7*) showed significantly higher methylation in cancer ($P < 0.05$, *t*-test). *POU4F2* tended to show higher methylation in cancer ($P = 0.07$, *t*-test). **(B)** Heatmap for methylation status of the 54 analyzed samples. Among the 34 papillary thyroid cancer samples, 26 were aberrant methylation(+) in at least one of six genes, while eight had no methylation. In **Table 4**, the number of cancer samples with aberrant methylation ($n = 26$) and the number of samples without aberrant methylation ($n = 8$) refer to these data.

Table 4 | Aberrant methylation and clinicopathological features.

Clinical features	All Cases ($n = 34$)	Aberrant Methylation(+) ($n = 26$, Figure 7B)	Aberrant Methylation(−) ($n = 8$, Figure 7B)	*P*-values
SEX				
Male/female	11/23	10/16	1/7	0.17 (Fisher)
AGE (YEAR)				
Mean ± SE	56.0 ± 2.7	57.2 ± 3.1	52.4 ± 4.9	0.45 (*t*-test)
TUMOR SIZE (mm)				
Mean ± SE	26.2 ± 2.6	28.3 ± 3.3	20.1 ± 2.0	0.06 (*t*-test)
NUMBER OF LYMPH NODES WITH METASTASIS				
Mean ± SE	2.6 ± 0.7	2.2 ± 0.6	3.3 ± 1.6	0.53 (*t*-test)
DISTANT METASTASIS				
(+)/(−)	0/34	0/26	0/8	1 (Fisher)
RECURRENCE				
(+)/(−)	5/28	3/22	2/6	0.37 (Fisher)
Unknown	1	1	0	
THYROGLOBULIN (ng/ml)				
Mean ± SE	104.6 ± 52.1	129.3 ± 68.6	30.5 ± 9.3	0.08 (*t*-test)
MUTATION OF BRAF/RAS ONCOGENES				
(+)/(−)	26/7	22/3	4/4	0.04* (Fisher)
Unknown	1	1	0	

*SE, standard error. P-values were calculated to compare methylation(+) group and methylation(−) groups and to analyze the correlation of methylation status to clinicopathological features. Fisher, calculated by Fisher's exact test. t-test, calculated by t-test. *P < 0.05, which is considered as statistically significant. Mutations of BRAF/RAS oncogenes are thus considered to correlate significantly with methylation(+) groups.*

recurrence and mortality ratios are significantly lower in stage I (15.4% and 1.7%, respectively) compared with more advanced tumors (22% and 15.8% in stage II, 46.4% and 30% in stage III, and 66.7% and 60.9% in stage IV, respectively) (Loh et al., 1997). Molecular diagnostic markers are still not used, although their development is anticipated (McLeod et al., 2013). Although aberrant methylation was not significantly associated with lymph node metastasis, distant metastasis, or recurrence in analysis of the 34 cancer samples in this study, further study should be performed using larger set of clinical samples for comparison of aberrant methylation, gene mutation status, and prognosis.

As for detected genes, *TLX3* (*HOX11L2*) is a transcription factor highly expressed in T-cell leukemia (Baak et al., 2008), and its aberrant methylation was observed in cisplatin-resistant bladder cancer (Tada et al., 2011). *SHOX2* is a member of the homeobox gene family, and is reported to relate to a short-stature phenotype of Turner syndrome (Clement-Jones et al., 2000). DNA methylation of *SHOX2* was suggested to be a biomarker for diagnosis of lung cancer based on bronchial aspirates (Schmidt et al., 2010). *HOXA7* is also a transcription factor belonging to the homeobox gene family that regulates gene expression, morphogenesis, and differentiation (La Celle and Polakowska, 2001). *POU4F2* is one of POU family genes with Pit-Oct-Unc domain, and is a transcription factor with a role in cell identity and regulation

of nerve cell or retinal development (Weishaupt et al., 2005). PHKG2 is the gamma subunit of phosphorylase kinase, containing the active site of the enzyme. Phosphorylase kinase-deficient liver glycogenosis can be caused by mutations of phosphorylase kinase subunits, *PHKA2*, *PHKB*, or *PHKG2*, but *PHKG2* mutation was reported to cause a severe phenotype of this disease (Burwinkel et al., 2003). *HIST1H3J* encodes a member of histone H3 family, and is found in the small histone gene cluster on chromosome 6p22-p21.3 (NCBI gene data bank). If the role of histone modifications is known to affect the regulation of gene expression, less is known about the possible direct involvement of histones, an H3 variant in the present case, in thyroid tumorigenesis. Further investigation is necessary to clarify tumorigenic roles of these genes and their methylation, in papillary thyroid cancer and other types of cancer (Schmidt et al., 2010; Tada et al., 2011).

In summary, 25 new genes were found to be frequently methylated in papillary thyroid cancer. There might be subsets of papillary thyroid cancer hardly methylated and preferentially methylated, and aberrant methylation of these genes correlates *a priori* to *BRAF*/*RAS* oncogene mutation in papillary thyroid cancer.

AUTHOR CONTRIBUTIONS

Yasuko Kikuchi, Koichi Yagi, and Keisuke Matsusaka performed the experiments. Eiichi Tsuji and Toshihisa Ogawa prepared clinical samples and information. Junichi Kurebayashi established and supplied cell lines. Yasuko Kikuchi, Shingo Tsuji and Atsushi Kaneda analyzed and interpreted the data. Yasuko Kikuchi and Atsushi Kaneda wrote the manuscript. Toshihisa Ogawa, Hiroyuki Aburatani, and Atsushi Kaneda supervised the study.

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MicroRNAs in Barrett's esophagus: future prospects

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Esophageal adenocarcinoma is an aggressive malignancy with a poor prognosis. In Western countries, the incidence of esophageal adenocarcinoma has increased dramatically in the last three decades. To improve patient survival and reduce disease burden, early-stage detection, or better yet, preventing the progression of esophageal adenocarcinoma from its premalignant lesions, constitute the best short-term options. Barrett's esophagus is histologically characterized by the replacement of the normal stratified squamous epithelium of the esophagus with a columnar epithelium with intestinal differentiation (Matsuzaki et al., 2010, 2011). Barrett's esophagus is considered to be a complication of gastroesophageal reflux disease and a precursor lesion of esophageal adenocarcinoma. It is generally believed that the progression of Barrett's esophagus involves a series of histological changes: non-dysplastic Barrett's metaplasia, low-grade dysplasia, high-grade dysplasia, and ultimately, adenocarcinoma. Although these features justify endoscopic surveillance for the premalignant stages, patients with Barrett's esophagus show an absolute annual risk of only 0.12% for the development of esophageal adenocarcinoma (Hvid-Jensen et al., 2011). Therefore, recommending the invasive and expensive conventional endoscopic screening procedure is deemed controversial. In fact, Corley et al. reported that, within a large community-based population, endoscopic surveillance of Barrett's esophagus was not associated with a substantial decrease in the risk of death from esophageal adenocarcinoma (Corley et al., 2013). Thus, identification of better risk stratification biomarkers to determine the risk of progression from Barrett's esophagus to esophageal adenocarcinoma may improve disease outcome and make patient management more cost-efficient.

MicroRNAs (miRNAs) are a class of small non-coding endogenous RNAs, 18–25 nucleotides in length, and are capable of simultaneous regulation of genes by binding to target mRNAs, resulting in mRNA degradation or translational inhibition. miRNAs participate in many essential biological processes, including proliferation, differentiation, apoptosis, necrosis, autophagy, and stress responses (Saito et al., 2011b, 2012a). miRNAs have also been shown to play a potential role in cancer pathogenesis through their functions as oncogenes or tumor suppressors, depending on their gene targets (Saito et al., 2009a, 2011a; Nishizawa and Suzuki, 2013). Compared to mRNAs, miRNAs are less numerous in humans and have been proposed to act as better biomarkers by virtue of their small size, greater stability, and capability of regulating hundreds of mRNAs. Therefore, miRNAs profiling could improve the risk stratification for the progression of Barrett's esophagus to esophageal adenocarcinoma.

MiRNAs can be profiled on a genome-wide scale using array or sequencing technologies. However, very few studies have been conducted to identify miRNAs as prognostic biomarkers for the progression of Barrett's esophagus to adenocarcinoma. Although several cross-sectional studies using comprehensive array analysis have been reported (Feber et al., 2008; Kan et al., 2009; Yang et al., 2009; Fassan et al., 2011; Leidner et al., 2012; Wu et al., 2013), their results have proved controversial. They compared the expression of miRNAs across different types of histological specimens such as Barrett's esophagus, low-grade dysplasia, high-grade dysplasia, and esophageal adenocarcinoma, and reported that a substantial number of miRNAs show differential expression in esophageal tissues (Sakai et al., 2013). Indeed, they might be useful in revealing certain mechanisms underlying carcinogenesis. But, they might be difficult to identify risk stratification biomarkers. We should think about much better research strategies.

Recently, two nice studies were reported to identify risk stratification biomarkers for Barrett's esophagus: one prospective study and one cross-sectional study. First, Revilla-Nuin et al. have reported a set of miRNAs associated with this progression and provided further validation in two groups of patients with Barrett's esophagus, who either developed or did not develop adenocarcinoma, over a course of 5 years (Revilla-Nuin et al., 2013). Among 24 patients with Barrett's esophagus, 7 patients progressed to adenocarcinoma while the other 17 did not. Four miRNAs (*miR-192*, *miR-194*, *miR-196a*, and *miR-196b*) were found to show significantly higher expression in patients with progression to esophageal adenocarcinoma than in patients who did not show disease progression. Second, Saad et al. conducted a notable comprehensive microarray profiling for identifying the specific miRNA signature associated with esophageal adenocarcinoma (Saad et al., 2013). They analyzed 13 samples from isolated Barrett's esophagus, 10 from Barrett's esophagus adjacent to high-grade dysplasia, 17 from high-grade dysplasia, and 34 from esophageal adenocarcinoma tissue. They

identified that *miR-21*, *miR-31*, *miR-192*, and *miR-194* were upregulated in Barrett's esophagus adjacent to high-grade dysplasia lesions as compared to isolated Barrett' esophagus. In addition, these 4 miRNAs were upregulated in a progressive manner through the Barrett's metaplasia-dysplasia-adenocarcinoma sequence. More importantly, this study provided findings for Barrett's esophagus for two groups: isolated Barrett's esophagus vs. Barrett's esophagus adjacent to high-grade dysplasia. The limitations of both two papers include the very small sample size. Larger prospective multi-institutional studies are warranted to confirm this result. Another criticism against the studies using comprehensive microarray analysis is that these could not provide the insights how miRNAs may exert their effects (Saito et al., 2009b, 2012b, 2013).

Since clinical predictors of increased risk of esophageal adenocarcinoma, namely, the length of Barrett's esophagus, male gender, older age, current tobacco smoking, alcohol consumption, central obesity, and bile reflux, have been established, the association between the expression levels of miRNA in Barrett's esophagus and these clinical risk factors would require further investigation. We had recently reported that expression levels of *miR-221* and *miR-222* increased when cultured esophageal epithelial cells were exposed to bile acids. *miR-221* and *miR-222* are known to specifically target p27Kip1, which in turn inhibits the proteasomal protein degradation of CDX2 (caudal-related homolog 2) (Matsuzaki et al., 2013). Furthermore, *miR-221* and *miR-222* expressions are higher in esophageal adenocarcinoma than in the surrounding Barrett's esophagus. We also confirmed that the levels of p27Kip1 and CDX2 were lower in areas of esophageal adenocarcinoma than in those of Barrett's esophagus. Thus, we showed that the degradation of CDX2 was enhanced by upregulation of *miR-221* and *miR-222* on exposure to bile acids. Although bile acids are known to induce DNA damage, resistance to apoptosis through NF-κB activation, and resistance to autophagy (Fang et al., 2013), the association between bile acids and miRNA expression has never been reported except for our results (Masaoka and Suzuki, 2014). In this way, clinical epidemiological information would be important and useful to reveal novel insights of miRNA in the progression of Barrett's esophagus to adenocarcinoma.

In conclusion, on the basis of clinical importance, better risk stratification biomarkers to determine the risk of progression from Barrett's esophagus to esophageal adenocarcinoma are expected. We should deepen our knowledge of miRNA using clinical materials, hopefully with more prospective approach. The fusion of basic science and clinical science research would also be required for identifying the upstream regulation and the downstream targets of miRNAs and understanding their mode of action. These will facilitate the development of miRNA-based prevention or therapeutic strategies for esophageal adenocarcinoma.

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Epigenetic alteration and microRNA dysregulation in cancer

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MicroRNAs (miRNAs) play pivotal roles in numerous biological processes, and their dysregulation is a common feature of human cancer. Thanks to recent advances in the analysis of the cancer epigenome, we now know that epigenetic alterations, including aberrant DNA methylation and histone modifications, are major causes of miRNA dysregulation in cancer. Moreover, the list of miRNA genes silenced in association with CpG island hypermethylation is rapidly growing, and various oncogenic miRNAs are now known to be upregulated via DNA hypomethylation. Histone modifications also play important roles in the dysregulation of miRNAs, and histone deacetylation and gain of repressive histone marks are strongly associated with miRNA gene silencing. Conversely, miRNA dysregulation is causally related to epigenetic alterations in cancer. Thus aberrant methylation of miRNA genes is a potentially useful biomarker for detecting cancer and predicting its outcome. Given that many of the silenced miRNAs appear to act as tumor suppressors through the targeting of oncogenes, re-expression of the miRNAs could be an effective approach to cancer therapy, and unraveling the relationship between epigenetic alteration and miRNA dysregulation may lead to the discovery of new therapeutic targets.

Keywords: microRNA, tumor suppressor, oncomir, CpG island methylation, histone modification, biomarker, EZH2

INTRODUCTION

MicroRNAs (miRNAs) are endogenous, small, non-coding single-stranded RNAs about 22 nucleotides in length, which function at the post-transcriptional level as negative regulators of gene expression (He and Hannon, 2004). Each miRNA negatively regulates its target genes in one of two ways, depending on the degree of complementarity between itself and its target messenger RNAs (mRNAs). miRNAs that bind to mRNA sequences with perfect or nearly perfect complementarity induce the RNA-mediated interference (RNAi) pathway, in which mRNA transcripts are cleaved by a miRNA-associated RNA-induced silencing complex (miRISC). This mechanism is mainly observed in plants, though miRNA-directed mRNA cleavage does occur in animals. Most animal miRNAs are thought to act by binding to imperfectly complementary sites within the 3′ untranslated regions (UTRs) of target mRNAs, thus inhibiting the initiation of translation via the miRISC.

Annotation of their genomic locations suggests that many miRNA genes are located within intergenic regions, though they are also found within exonic and intronic regions in either the sense or antisense orientation. Like genes encoding proteins, miRNA genes are mainly transcribed by RNA polymerase II. They are initially transcribed as large precursors, called primary miRNAs (pri-miRNAs), which may encode multiple miRNAs in a polycistronic arrangement. Pri-miRNAs are then processed by the RNase III enzyme Drosha and its cofactor DGCR8/Pasha to produce ∼70-nt hairpin-structured second precursors, called pre-miRNAs. The pre-miRNAs are transported to the cytoplasm by the nuclear export protein Exportin-5 (XPO5), after which they are processed by another RNase III enzyme, DICER, to generate mature miRNA products.

Sequences of miRNAs are highly conserved among species, and play critical roles in a variety of biological processes, including cell proliferation, development, differentiation, and apoptosis. In addition, subsets of miRNAs are thought to act as tumor suppressor genes or oncogenes, and their dysregulation is a common feature of human cancers (Esquela-Kerscher and Slack, 2006; Croce, 2009). More specifically, expression of miRNAs is generally downregulated in tumor tissues, as compared to corresponding healthy tissues, which suggests some miRNAs behave as tumor suppressors in some tumors. Although the mechanism underlying the alteration of miRNA expression in cancer is still not fully understood, recent studies have shown that cancer affects multiple mechanisms involved in regulating miRNA levels. For example, a significant number of miRNAs are located within cancer-associated genomic regions or in fragile sites (Calin et al., 2004). The first report of altered miRNA expression in cancer was related to the frequent chromosomal deletion and downregulated expression of miR-15 and miR-16, two miRNAs thought to target the antiapoptotic factor B cell lymphoma 2 (BCL2) in chronic lymphocytic leukemia (CLL; Calin et al., 2002). More recent studies indicate that genetic mutations affecting proteins involved in the processing and maturation of miRNA, such as TARBP2 and XPO5, can also lead to overall reductions in miRNA expression (Melo et al., 2009, 2010). In addition, epigenetic alterations, including aberrant DNA methylation and histone modifications, appear to be a major mechanism by which the normal patterns of miRNA expression are disrupted in cancer. In this review, we will highlight the contribution made by epigenetic alteration of miRNAs to cancer, and discuss their clinical application as biomarkers and therapeutic targets.

IDENTIFICATION OF EPIGENETICALLY DYSREGULATED miRNAs IN CANCER

The first evidence that epigenetic mechanisms are involved in silencing miRNAs in cancer came from a pharmacological unmasking experiment. Using a miRNA microarray, Saito et al. (2006) analyzed the expression profiles of miRNAs in T24 human bladder cancer cells and LD419 human normal fibroblasts treated with or without the DNA methyltransferase (DNMT) inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) and the histone deacetylase (HDAC) inhibitor 4-phenylbutyric acid (4-PBA). Among the genes upregulated in the cancer cells was miR-127, which was embedded within a CpG island and was upregulated in association with DNA demethylation, acetylation of histone H3 and trimethylation of histone H3 lysine 4 (H3K4me3), which are marks of active transcription. Experimental evidence confirmed that the proto-oncogene BCL6 is a target of miR-127, suggesting miR-127 acts as a tumor suppressor (Saito et al., 2006).

As with protein-coding genes, epigenetic regulation of miRNA genes is tightly associated with histone modification (**Figure 1**). As mentioned, H3K4me3 and acetylation of histone H3 lysine 9/14 are hallmarks of active miRNA gene promoters in embryonic stem cells and in cancer cells (Marson et al., 2008; Ozsolak et al., 2008; Suzuki et al., 2011). By contrast, di- or trimethylation of histone H3 lysine 9 (H3K9me2 or H3K9me3) and trimethylation of lysine 27 (H3K27me3) are marks of repression. For instance, a combination of chromatin immunoprecipitation (ChIP)-on-chip and miRNA microarray analyses in prostate cancer cells revealed that miRNA expression correlates positively with H3K4me3 and correlates inversely with H3K27me3 in miRNA promoter regions (Ke et al., 2009). In addition, genome-wide screening for miRNA genes with reduced levels of H3K4me3 and increased levels of H3K9me2 led to the identification of 13 miRNA genes, including the miR-124 family, miR-9 family, and miR-34b/c, that are epigenetically silenced in acute lymphoblastic leukemia (ALL; Roman-Gomez et al., 2009). To assess genome-wide histone modifications, we recently performed deep sequencing (ChIP-seq) in colorectal cancer (CRC) cells and identified the putative promoter regions of 174 pri-miRNA genes (Suzuki et al., 2011). By searching for miRNAs that showed upregulated expression and increases in H3K4me3 marks upon DNA demethylation, we identified 37 miRNA genes as potential targets of epigenetic silencing in CRC cells.

Epigenetically silenced miRNA genes were also identified through genome-wide DNA methylation analysis. For instance, methylation microarray analysis using the Infinium BeadChip revealed miR-10b to be a target of DNA methylation in gastric cancer (GC; Kim et al., 2011). In addition, Yan et al. (2011) performed a genome-wide methylome analysis entailing deep sequencing of MBD (methylated DNA binding domain)-isolated DNA in HCT116 cells, and identified a number of methylated genes, including miR-941, miR-1237, and miR-1247. And Baer et al. (2012) carried out an integrative analysis of genome-wide DNA methylation and histone modification (H3K4me3) in CLL and identified 128 miRNAs that carried aberrant DNA methylation at their promoters. Interestingly, of those 128 miRNA promoters, 38 exhibited hypermethylation, while 90 showed hypomethylation, which are indicative of epigenetically silenced and activated miRNAs, respectively. In fact, the hypermethylated

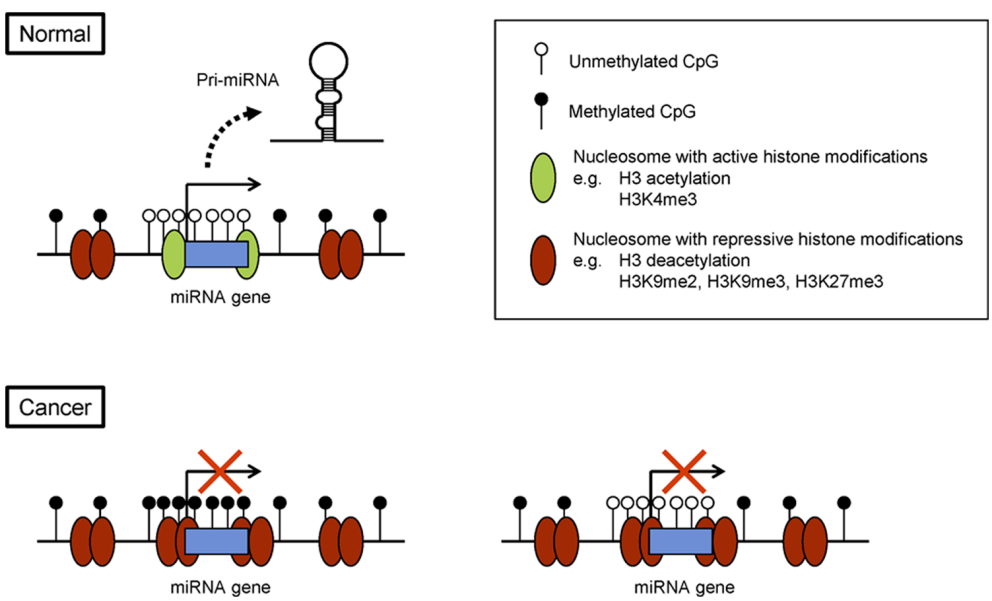

FIGURE 1 | Epigenetic silencing of miRNA genes in cancer. Example of a miRNA gene with its CpG island. In normal cells, the CpG island is unmethylated, and the chromatin is associated with active histone modifications. In cancer cells, CpG island hypermethylation and repressive histone marks lead to epigenetic silencing of the miRNA gene (left). Alternatively, some miRNA genes are silenced through repressive histone modifications, rather than DNA methylation (right).

regions included a number of well-defined epigenetically silenced miRNA genes, including miR-9-2, miR-124-2, and miR-129-2, while the hypomethylation was accompanied by upregulation of several miRNAs, including miR-21, miR-34a, and miR-155.

ABERRANT DNA METHYLATION OF miRNA GENES IN CANCER

Among the rapidly growing list of miRNAs that are now known to be aberrantly methylated in cancer, many are downregulated in association with CpG island hypermethylation, while some are upregulated via hypomethylation of their CpG island. Here we describe well-characterized miRNA genes showing aberrant DNA methylation in cancer.

Epigenetic silencing of miR-124 family genes was first discovered in CRC (Lujambio et al., 2007), and they are now known to be methylated in several other types of malignancy, including ALL (Agirre et al., 2009), non-Hodgkin's lymphoma (Wong et al., 2011a), and liver (Furuta et al., 2010), pancreatic (Wang et al., 2013), renal (Gebauer et al., 2013), and cervical cancer (Wilting et al., 2010). Within the human genome, three independent loci (miR-124-1, miR-124-2, and miR-124-3) encode identical mature forms of miR-124, and all are associated with CpG islands, which may be targets of hypermethylation in cancer (Lujambio et al., 2007). miR-124 exerts its tumor suppressor function by targeting cyclin-dependent kinase 6 (CDK6), and epigenetic silencing of miR-124 reportedly results in CDK6 activation and Rb phosphorylation (Lujambio et al., 2007; Agirre et al., 2009). In ALL, miR-124 methylation is associated with higher recurrence and mortality rates, and may be an independent prognostic factor for disease-free and overall survival (Agirre et al., 2009). miR-124 family genes are also frequently methylated in the gastric mucosa of *Helicobacter pylori*-positive healthy individuals, suggesting their methylation could be induced by chronic inflammation (Ando et al., 2009).

Members of the miR-34 gene family (miR-34a, miR-34b, and miR-34c) are direct targets of p53, and their ectopic expression in cancer cells induces cell cycle arrest and apoptosis (Bommer et al., 2007; He et al., 2007). Within the human genome, miR-34a is located on chromosome 1p36, while miR-34b and miR-34c are co-transcribed from a single transcription unit on chromosome 11q23. The promoters of both genes are targets of CpG island hypermethylation in multiple malignancies including oral (Kozaki et al., 2008), esophageal (Chen et al., 2012), gastric (Suzuki et al., 2010), colorectal (Toyota et al., 2008), lung (Wang et al., 2011b), breast and renal cancer (Lodygin et al., 2008; Vogt et al., 2011), and hematological malignancies (Roman-Gomez et al., 2009; Wong et al., 2011b). Methylation of miR-34b/c has also been linked to cancer metastasis (Lujambio et al., 2008) and invasion (Watanabe et al., 2012). In addition, methylation-associated silencing of miR-34c was recently shown to promote self-renewal and epithelial–mesenchymal transition (EMT) in breast tumor-initiating cells (Yu et al., 2012). These findings, as well as their contribution to the p53 network, strongly imply that miR-34 family members act as tumor suppressors in cancer. Introduction of miR-34b/c into cancer cells leads to the downregulation of candidate target genes, including MET, CDK4, cyclin E2 (CCNE2), and MYC (Lujambio et al., 2008; Toyota et al., 2008). Likewise, restoration of endogenous miRNA expression through demethylation also downregulates target genes, suggesting miRNAs could be important targets for epigenetic cancer therapy (Toyota et al., 2008).

The CpG islands of miR-9 family genes (miR-9-1, miR-9-2, and miR-9-3) are also frequently methylated in various types of malignancies, including ALL (Roman-Gomez et al., 2009) and colorectal (Bandres et al., 2009), breast (Lehmann et al., 2008), pancreatic (Omura et al., 2008), and GCs (Tsai et al., 2011). Moreover, a screen for miRNA gene methylation in metastatic cancer cell lines identified miR-9 family genes as being methylated (Lujambio et al., 2008). Consistent with that finding, methylation of miR-9-1 is reportedly associated with lymph node metastasis in CRC (Bandres et al., 2009), while methylation of miR-9-1 and miR-9-3 correlates with metastatic recurrence of renal cell carcinoma (Hildebrandt et al., 2010). miR-9 has been shown to target fibroblast growth factor receptor 1 (FGFR1) and CDK6 in ALL (Rodriguez-Otero et al., 2011) and caudal-type homeobox 2 (CDX2) in GC (Rotkrua et al., 2011), suggesting a tumor-suppressive function.

The miR-200 gene family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 encode key regulators of EMT that act by directly targeting zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2, which are transcriptional repressors that downregulate E-cadherin (CDH1; Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008). Within the human genome, miR-200 family genes are grouped into two polycistronic units, miR-200b/200a/429 and miR-200c/141, located on chromosomes 1 and 12, respectively (Davalos et al., 2012). In normal mammary epithelial cells and fibroblasts, expression of miR-200 family and miR-205 genes is regulated by DNA methylation, histone modifications, or a combination of the two (Vrba et al., 2010), but aberrant DNA methylation leads to the silencing of these miRNAs in cancer (Ceppi et al., 2010; Neves et al., 2010; Wiklund et al., 2011). For instance, methylation of miR-200c/141 is tightly correlated with the invasive capacity of breast cancer cells (Neves et al., 2010). Similarly, in non-small cell lung cancer, promoter methylation is associated with loss of miR-200c expression, which is in turn associated with poor differentiation, lymph node metastasis, and weaker E-cadherin expression (Ceppi et al., 2010). Davalos et al. (2012) demonstrated that the upstream CpG islands of both units (miR-200b/200a/429 and miR-200c/141) are unmethylated in cancer cells with epithelial features, but are both methylated and silenced in transformed cells with mesenchymal characteristics.

In addition to its therapeutic implications, miRNA gene methylation could be a useful molecular marker for detecting cancer and/or predicting its outcome. For instance, the CpG island of miR-34b/c is methylated in more than 90% of primary CRCs, and methylation was detected in 75% of fecal specimens from CRC patients and in 16% of specimens from high-grade dysplasia patients, suggesting miR-34b/c methylation could be a useful feces-based screening marker (Kalimutho et al., 2011). It was also recently shown that miR-34a methylation and high levels of c-MET and β-catenin expression may be powerful predictive markers of liver metastasis in CRC (Siemens et al., 2013). In addition, miR-34b/c methylation was found to be elevated in the background

gastric mucosa of multiple GC patients (Suzuki et al., 2010), and a subsequent study revealed that miR-34b/c methylation could be a marker for predicting the risk of metachronous GC (Suzuki et al., 2013). miRNA gene methylation is also detectable in urine specimens, and could be a useful marker of urinary tract cancer. A recent screening for epigenetically silenced miRNAs in bladder cancer cells identified methylation of four miRNA genes (miR-137, miR-124-2, miR-124-3, and miR-9-3), and their methylation in urinary DNA was found to be a useful biomarker of bladder cancer (Shimizu et al., 2013).

Many miRNA genes are reportedly downregulated in association with DNA hypermethylation in cancer, but some are epigenetically activated via DNA hypomethylation. As mentioned, a recent comprehensive analysis of miRNA in CLL revealed that approximately 60% of aberrantly methylated miRNA genes exhibited hypomethylation (Baer et al., 2012). For instance, the CpG island of let-7a-3 is heavily methylated in normal cells but is hypomethylated in lung adenocarcinoma, leading to its elevated expression (Brueckner et al., 2007). In lung cancer cells, let-7a-3 exerts oncogenic effects through actions on several genes involved in cell proliferation, adhesion, and differentiation. In addition, miR-200a and miR-200b are overexpressed in pancreatic cancer due to their hypomethylation, and their elevation in the serum of pancreatic cancer patients means they could potentially serve as diagnostic biomarkers (Li et al., 2010). miR-196 family genes (miR-196a and miR-196b) are located within the HOX gene cluster and are often overexpressed in tumors, which is indicative of their oncogenic functions (Luthra et al., 2008; Maru et al., 2009; Popovic et al., 2009; Guan et al., 2010). miR-196b is embedded within a CpG island, and its overexpression in GC is associated with its hypomethylation (Tsai et al., 2010).

Recently, Fornari et al. (2012) reported that miR-519d is upregulated due to DNA hypomethylation in hepatocellular carcinoma (HCC). miR-519d belongs to the chromosome 19 miRNA cluster (C19MC), which is the largest miRNA cluster in the human genome. miRNAs in the C19MC are normally expressed specifically in placenta (Bentwich et al., 2005), but DNA demethylation leads to their re-expression in cancer cells, which is indicative of their epigenetic repression in healthy tissue (Tsai et al., 2009; Suzuki et al., 2011). Upregulation of miR-519d, which is observed in approximately 50% of HCCs, is positively associated with CpG island hypomethylation and wild-type p53 (Fornari et al., 2012). miR-519d is thought to act as an oncogenic miRNA (oncomir) through its targeting of p21, PTEN, AKT3, and TIMP2.

miRNA DYSREGULATION CAUSES ABERRANT DNA METHYLATION

Several lines of evidence support the idea that dysregulation of miRNAs can lead to aberrant DNA methylation in cancer. For instance, the miR-29 family (miR-29a, miR-29b, and miR-29c), which is downregulated in lung cancer, directly targets DNMT3A and DNMT3B (Fabbri et al., 2007; **Figure 2**). Ectopic expression of the miR-29 family in lung cancer cells restores expression of methylation-silenced tumor suppressor genes, including fragile histidine triad (FHIT) and WW domain containing oxidoreductase (WWOX). In addition, miR-143 is frequently downregulated in CRC cells, where it normally targets DNMT3A (Ng et al., 2009), and downregulated expression of miR-152 in HBV-related HCC correlates with increased expression of DNMT1 (Huang et al., 2010). Forced expression of miR-152 in liver cell lines reduces DNMT1 expression and global DNA methylation, whereas inhibition of miR-152 causes global DNA hypermethylation and increased methylation of the glutathione *S*-transferase pi 1 (GSTP1) and CDH1 promoter regions. Similarly, DNMT1 is targeted by miR-148a and miR-152 in cholangiocarcinoma cells, and their ectopic expression suppresses DNMT1 and induces expression of the tumor suppressor genes Ras association domain family 1A (RASSF1A) and p16 (Braconi et al., 2010). miR-342 was found to be downregulated in CRC cells, and restoration of its expression downregulated DNMT1 and reactivated expression of cancer-related genes through demethylation of their promoter regions (Wang et al., 2011a). miR-185 is downregulated in glioma cells in association with loss of heterozygosity (LOH), and its restoration reduces global DNA methylation and leads to re-expression of hypermethylated genes through targeting DNMT1 (Zhang et al., 2011b).

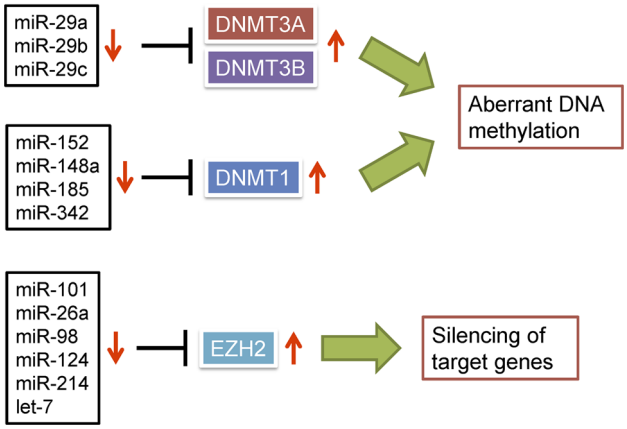

FIGURE 2 | Dysregulation of miRNAs is causally related to epigenetic alterations in cancer. miRNAs able to negatively regulate DNMTs or EZH2 are frequently downregulated in cancer, which leads to epigenetic silencing of target genes.

miR-34b was recently shown to target both DNMTs and HDACs in prostate cancer cells (Majid et al., 2013). As in other malignancies, miR-34b is silenced in association with CpG island methylation in prostate cancer, and low miR-34b expression is strongly associated with poor survival. Interestingly, ectopic expression of miR-34b in prostate cancer cells suppressed DNMTs and HDACs and induced partial demethylation and active chromatin modification of the endogenous miR-34b gene, which suggests a positive feedback loop. Collectively, these results indicate that dysregulation of specific miRNAs may be causally related to aberrant methylation of promoter CpG islands.

HISTONE MODIFICATIONS AND miRNA DYSREGULATION

It is now evident that histone modifications also play a major role in the dysregulation of miRNAs in cancer. For example, treating a breast cancer cell line with the HDAC inhibitor LAQ824 induced

upregulation or downregulation of a number of miRNAs within as little 5 h (Scott et al., 2006). Not only does this suggest the involvement of epigenetic mechanisms in the regulation of miRNAs in cancer cells, it also highlights the importance of secondary effects driven by miRNAs induced or downregulated through drug treatment. In addition, recent studies have shown that HDAC silences tumor-suppressive miRNAs in cancer. It is well documented that loss of miR-15a and miR-16 in CLL is associated with 13q loss; however, these miRNAs are also often downregulated in CLL samples without observable deletions in 13q, and Sampath et al. (2012) found that overexpression of HDACs (HDAC1, HDAC2, and HDAC3) is associated with downregulation of miR-15a, miR-16, and miR-29b. Furthermore, inhibition of the HDACs induced robust accumulation of active histone marks at the promoters of the miRNAs and increased their expression, which in turn led to downregulation of their target genes, BCL2 and MCL1. In another study, MYC interacted with HDAC3, which then colocalized to the promoters of miR-15a/miR-16-1 and their host gene DLEU2, resulting in MYC-induced suppression of these miRNAs in mantle cell lymphoma (Zhang et al., 2012a). And in HCC, upregulation of HDACs (HDAC1–3) was associated with repression of miR-449, which led to activation of the putative miR-449 target gene c-MET (Buurman et al., 2012).

In other settings, histone acetylation is involved in the activation of oncomirs in cancer. For example, miR-224 is commonly upregulated in HCC, and there is reportedly a positive correlation between miR-224 expression and histone acetylase protein EP300 in HCC tumors (Wang et al., 2012). It is well documented that the breast cancer susceptibility gene BRCA1 is involved in DNA damage repair and cell cycle regulation, but a recent study revealed an interesting link between BRCA1 and the epigenetic regulation of oncomirs. Chang et al. (2011) showed that wild-type BRCA1 epigenetically represses miR-155 by recruiting HDAC2 to the miR-155 promoter, while a BRCA1 R1699Q mutant relieves the repression and causes miR-155 to be overexpressed.

As mentioned, miRNA gene transcription is closely associated with histone modifications; thus some miRNA genes are silenced without DNA hypermethylation in cancer cells (**Figure 1**). For example, downregulation of miR-212 in lung cancer cells is reportedly associated with H3K9me2 and H3K27me3 but not DNA hypermethylation (Incoronato et al., 2011; **Figure 1**). miR-212 exerts a pro-apoptotic effect in lung cancer cells by targeting the anti-apoptotic gene PED, and inhibition of HDAC and the histone methyltransferase EZH2 strongly reactivates miR-212 expression in lung cancer cells. It was also recently found that miR-708 is repressed by H3K27me3 in metastatic breast cancer (Ryu et al., 2013). miR-708 targets neuronatin (NNAT), a regulator of intracellular Ca^{2+}, and silencing miR-708 leads to elevation of intracellular Ca^{2+} levels and increased cell migration and metastasis.

miRNA DYSREGULATION CAUSES ABERRANT HISTONE MODIFICATIONS

Dysregulation of miRNAs can also lead to aberrant histone modifications. EZH2 is a member of the polycomb group (PcG) of proteins, which are key regulators that silence numerous developmental genes (Schuettengruber et al., 2007). EZH2 functions as a catalytic subunit of polycomb repressive complex 2 (PRC2), which trimethylates H3K27. The available evidence suggests that EZH2 has oncogenic properties, and its overexpression in prostate and breast cancers promotes tumorigenesis, invasiveness and metastasis (Varambally et al., 2002; Kleer et al., 2003). Varambally et al. (2008) reported that EZH2 is a target of miR-101, and genomic loss of miR-101 is an important cause of EZH2 overexpression in cancer. Reduced expression of miR-101 and upregulation of EZH2 occur in parallel during the progression of prostate cancer, and genomic loss of miR-101 is more frequently seen in metastatic disease than localized cancers. Moreover, the loss of miR-101 and resultant overexpression of EZH2 appears to alter the global chromatin structure in cancer (Friedman et al., 2009). The inverse association between miR-101 and EZH2 has now been seen in bladder, gastric, lung, and renal cancer (Friedman et al., 2009; Wang et al., 2010; Zhang et al., 2011a; Sakurai et al., 2012). In addition, several other miRNAs, including miR-26a (Wong and Tellam, 2008), miR-98 (Alajez et al., 2010), miR-124 (Zheng et al., 2012), miR-144 (Guo et al., 2013), miR-214 (Derfoul et al., 2011), and let-7 (Kong et al., 2012) are also reported to negatively regulate EZH2 (**Figure 2**). Thus, dysregulation of miRNAs appears to be one of the major causes of EZH2 overexpression in cancer.

Overexpression of EZH2 also leads to the silencing of multiple miRNAs in cancer. It was recently demonstrated that EZH2 is frequently upregulated in primary HCCs, and miRNA expression profiling in HCC cells with EZH2-knockdown revealed that a set of miRNAs, including miR-139-5p, miR-125b, let-7c, miR-101, and miR-200b, are epigenetically suppressed by EZH2 in HCC (Au et al., 2012). Interestingly, miR-200b reportedly targets another PRC2 subunit, SUZ12, in breast cancer stem cells (Iliopoulos et al., 2010), suggesting a possible feedback loop between EZH2 overexpression and miRNA silencing in cancer. In another study, Cao et al. (2011) demonstrated that in prostate and breast cancer cell lines, EZH2 represses a set of miRNAs (miR-181c, miR-181b, miR-200b, miR-200c, and miR-203), which in turn negatively regulate the PRC1 subcomponents BMI1 and RING2. The inverse correlation between miRNA and PRC protein levels were further confirmed in prostate cancer tissues. These results are indicative of an integral regulatory axis involving PRC1, PRC2, and the epigenetic silencing of miRNAs in cancer.

Another recent study demonstrated the involvement of EZH2 and HDAC3 in MYC-mediated miRNA repression. In aggressive B cell lymphoma, miR-29a is repressed by MYC within a co-repressor complex that also includes HDAC3 and EZH2 (Zhang et al., 2012b). Interestingly, MYC contributes to EZH2 upregulation through repression of miR-26a, which targets EZH2, while EZH2 upregulates MYC by inhibiting miR-494, which targets MYC. It thus appears a positive MYC-miRNA-EZH2 feedback loop may mediate persistent overexpression of MYC and EZH2. Combined inhibition of HDAC3 and EZH2 induced restoration of miR-29 and suppressed lymphoma cell growth, suggesting the MYC–EZH2–miRNA axis could be a promising target for epigenetic therapy in B cell lymphoma.

CONCLUDING REMARKS

In this review, we highlighted the relationship between epigenetic alteration of miRNAs and cancer. Aberrant DNA methylation

commonly underlies miRNA dysregulation in cancer, and methylation of a subset of miRNA genes may be a useful biomarker for detecting cancer and/or predicting clinical outcome. Alteration of the histone modification pattern also leads to abnormal miRNA expression. In addition, recent findings suggest that miRNA dysregulation is causally related to aberrant DNA methylation and histone modifications that leads to genome-wide epigenetic abnormalities. It is anticipated that additional study of the relationship between epigenetic regulation and miRNAs will lead to the discovery of new biomarkers as well as therapeutic targets.

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The role of microRNAs in the regulation of cancer stem cells

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Cancer stem cells (CSCs) have been reported in many human tumors and are proposed to drive tumor initiation and progression. CSCs share a variety of biological properties with normal somatic stem cells such as the capacity for self-renewal, the propagation of differentiated progeny, and the expression of specific cell surface markers and stem cell genes. However, CSCs differ from normal stem cells in their chemoresistance and tumorigenic and metastatic activities. Despite their potential clinical importance, the regulation of CSCs at the molecular level is not well-understood. MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs that play an important role in the regulation of several cellular, physiological, and developmental processes. Aberrant miRNA expression is associated with many human diseases including cancer. miRNAs have been implicated in the regulation of CSC properties; therefore, a better understanding of the modulation of CSC gene expression by miRNAs could aid the identification of promising biomarkers and therapeutic targets. In the present review, we summarize the major findings on the regulation of CSCs by miRNAs and discuss recent advances that have improved our understanding of the regulation of CSCs by miRNA networks and may lead to the development of miRNA therapeutics specifically targeting CSCs.

Keywords: microRNA, cancer stem cells (CSCs), tumor initiation, therapy resistance, metastasis

BACKGROUND

The CSC theory, which is based on the concept that cancer might arise from a rare population of cells with stem cell properties, was proposed approximately 150 years ago (Cohnheim, 1875; Wicha et al., 2006). Recent technological developments (flow cytometry analysis and cell sorting) and the establishment of new animal models have provided evidence supporting the CSC theory. Moreover, CSCs are resistant to conventional treatments and are therefore not only of academic interest, but may also be an important consideration in clinical practice. Therefore, a better understanding of the characteristics of CSCs and the identification of therapeutic agents capable of targeting the CSC population are critical issues. Cancer researchers have investigated protein-coding genes and products, including surface markers that are involved in the self-renewal and asymmetric cell division of CSCs. Recently, in addition to alterations in protein-coding genes, abnormalities in non-coding RNAs [miRNAs and long intergenic non-coding RNAs] have been observed in various types of cancers and have been shown to play important roles in the regulation of CSC properties such as asymmetric cell division, tumorigenicity, and drug resistance. In the present review, we discuss the general features of CSCs and the role of miRNAs in the regulation of CSC properties, and summarize the current therapeutic strategies targeting miRNAs for CSC therapy.

BIOGENESIS AND FUNCTIONS OF miRNAs

miRNAs are 21–25 nucleotides long, non-coding RNAs that regulate gene expression at the post-transcriptional level by binding to the 3′-untranslated regions (3′UTRs) or the open reading frames of target genes, leading to the degradation of target mRNAs or repression of mRNA translation (Iorio and Croce, 2012). miRNAs are transcribed for the most part by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-miRNA), and are processed in the nucleus by RNase III Drosha into 70–100 nucleotide long precursor miRNAs (pre-miRNAs) in combination with cofactors such as DGCR8, an evolutionarily conserved protein that interacts with proline-rich peptides through its WW domain (Gregory et al., 2004; Lee et al., 2004) (**Figure 1**). *DGCR8* is located on chromosome region 22q11.2, whose heterozygous deletion results in the most common human genetic deletion syndrome, known as DiGeorge syndrome. The clinical symptoms of the disease are highly variable and in approximately 75% of patients, congenital heart defects are observed (Shiohama et al., 2003; Yamagishi and Srivastava, 2003). The product of pri-miRNA cleavage, the pre-miRNA, is exported to the cytoplasm by exportin-5, a member of the Ran-dependent nuclear transport receptor family (Lee et al., 2004) and further cleaved in a complex composed of RNase III Dicer and the transactivating response RNA- binding protein (TRBP) into a miRNA:miRNA* complex. While one of the two strands is selected as a guide strand, the complementary strand (miRNA*) is usually degraded (Iorio and Croce, 2012). miRNA* was originally considered to have no function and to be degraded; however, recent evidence suggests that it can be used as a functional strand and may play significant biological roles (Uchino et al., 2013; Yang et al., 2013).

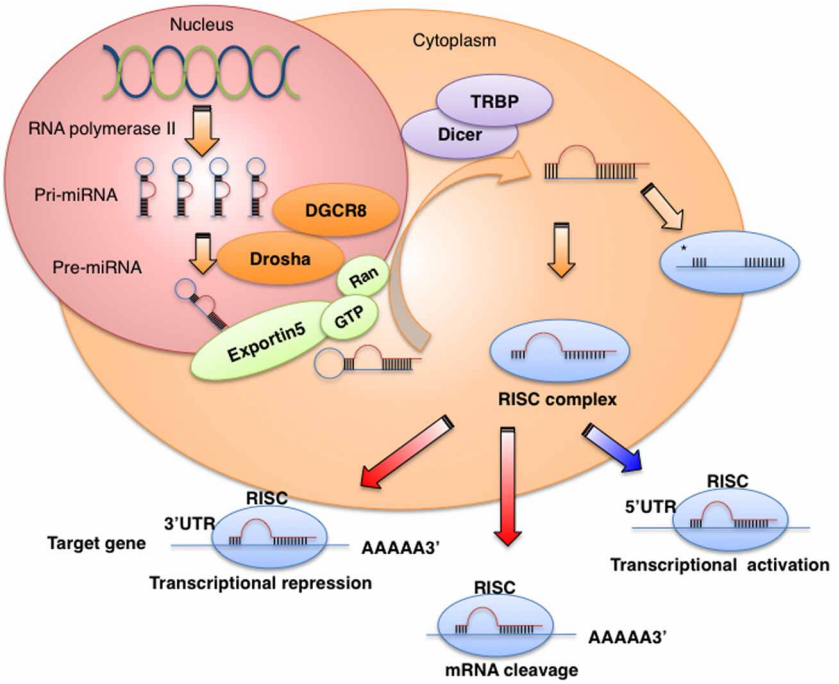

FIGURE 1 | miRNA biogenesis and function. miRNAs are transcribed by RNA polymerase II or III as pri-miRNA, and are processed in the nucleus by Drosha-DGCR8 into pre-miRNAs. The product of pri-miRNA cleavage, the pre-miRNA, is exported to the cytoplasm by exportin-5 and further cleaved in a complex composed of Dicer and TRBP. The functional strand of mature miRNA is incorporated into the RNA-induced silencing complex (RISC), which contains GW182 and Argonaute protein. As a part of this complex, the mature miRNA regulates gene expression by binding to partially complementary sequences in the 3′UTRs of target mRNAs, leading to mRNA degradation or translation inhibition.

The mature miRNA is incorporated into a complex known as the RNA-induced silencing complex (RISC), which contains the GW182 and Argonaute proteins. As a part of this complex, the mature miRNA regulates gene expression by binding to partially complementary sequences in the 3′UTRs of target mRNAs, leading to mRNA degradation or translation inhibition (Iorio and Croce, 2012). Several studies have reported that miRNAs also bind to the 5′UTR or the open reading frame (Orom et al., 2008; Mandke et al., 2012) and can promote the translation of their target genes under growth arrest conditions (Vasudevan et al., 2007). Recently, Nishi et al. showed that TNRC6A, a human GW182 paralog, shuttles Ago2 into the nucleus and the colocalization of Ago2-TNRC6A with miRNAs mediates gene silencing (Nishi et al., 2013).

MICRORNAs REGULATE PLURIPOTENCY AND DIFFERENTIATION

The discovery of two miRNAs, lin-4 and let-7, in *Caenorhabditis elegans* suggested that miRNAs are important regulators of embryonic development and stem cell functions in mammals (Lee et al., 1993; Pasquinelli et al., 2000; Reinhart et al., 2000). The function of miRNAs in mouse and human embryonic stem cells (ESCs) has been investigated using cells lacking Dicer1 and DGCR8, which are critical for miRNA biogenesis. Deletion of Dicer1 leads to embryonic lethality in mice (Bernstein et al., 2003) and DGCR8-deficient mouse ESCs show alterations in the regulation of the cell cycle and differentiation that are associated with failure to silence stemness markers, such as *Oct4*, *Rex1*, *Sox2*, and *Nanog*, as well as delayed expression of differentiation markers (Wang et al., 2007).

In a comparative transcriptome analysis, Dicer1-deficient mouse ESCs lacking miRNAs showed a significant increase in transcripts containing a GCACUU motif in the 3′UTR (Sinkkonen et al., 2008). This sequence is complementary to the AAGUGC seed sequence of the miR-290-295 cluster (miR-290, miR-291a, miR-292, miR-291b, miR-294, and miR-295) and the miR-302/367 cluster (miR-302a, miR-302b, miR-302c, miR-302d, and miR-367) in mouse ESCs. Using a similar approach, novel stem cell-specific miRNAs were initially identified in human ESCs. These miRNAs include two clusters: miR-302/367 and the miR-371 cluster (miR-372 and miR-373). The expression of the miR-371 cluster is downregulated before that of the miR-302/367 cluster, suggesting a temporal hierarchy in the duration of specific miRNA activity (Stadler et al., 2010; Kim et al., 2011). Members of the miR-302 family rescue the proliferation defects of DGCR8-mutant mouse ESCs (Wang et al., 2008) and reprogram human skin cancer cells into a pluripotent ESC-like state (Lin et al., 2008).

The Let-7 family is another critical regulator of ESC differentiation. Mature let-7 family members are essentially absent in ESCs and accumulate only upon ESC differentiation (Viswanathan et al., 2008). Melton et al. reported that whereas transfection of let-7c into wild-type cells had no effect on the expression of pluripotency genes, let-7c rescued the differentiation defect in DGCR8$^{-/-}$ cells by downregulating *Oct4*, *Sox2*, and *Nanog*

(Melton et al., 2010). Lin-28, a marker of undifferentiated ESCs, is also used to induce pluripotent stem cells (Yu et al., 2007b). A negative feedback loop between Lin-28 and let-7 family members precisely controls the levels of these miRNAs. Although Lin-28 regulates the expression of let-7 miRNAs by binding to the precursors and blocking their maturation, the let-7 family is highly expressed and targets Lin-28 mRNA in mouse differentiated cells and embryonic carcinoma cells (Yu et al., 2007b) (**Figure 2**). Members of the miR-34 family of miRNAs are direct targets of p53 and function as tumor suppressors, inhibiting reprogramming through the repression of pluripotency genes such as *Nanog*, *Sox2*, and *N-myc* (Choi et al., 2011) (**Figure 2**). Since the cell cycle regulator p21 also represses reprogramming efficiency, these findings suggest that p53 represses pluripotency via two distinct mechanisms. Evidence that let-7 and miR-34 family members are tumor suppressor miRNAs (Takamizawa et al., 2004; Johnson et al., 2005; Tazawa et al., 2007) suggests that stem cell-specific miRNAs play important roles in tumor initiation and development.

miRNA REGULATION IN CANCER

miRNAs play a crucial role in the progression of human cancer, and expression profiling in human malignancies has identified signatures associated with cancer development, progression, and prognosis (Liu et al., 2012; Volinia and Croce, 2013). Chromosomal regions coding for oncogenic miRNAs that are involved in the negative regulation of a tumor suppressor gene can be amplified in association with cancer development. This amplification would result in the upregulation of oncogenic miRNAs and silencing of tumor suppressor genes (He et al., 2005). On the other hand, miRNAs targeting oncogenes are often located in fragile site, where deletions or mutations can occur, leading to the reduction or loss of miRNAs and the overexpression of their target oncogenes. Dysregulation of miRNA expression affects processes associated with cancer progression such as the induction of anti-apoptotic activity, drug resistance, tissue invasion, and metastasis (Cimmino et al., 2005; Tavazoie et al., 2008; To et al., 2008). Recent evidence suggests that miRNAs are involved in tumor initiation through the regulation of CSC properties such as self-renewal ability, tumorigenicity and drug-resistance (Yu et al., 2007a; Shimono et al., 2009; Song et al., 2013a,b).

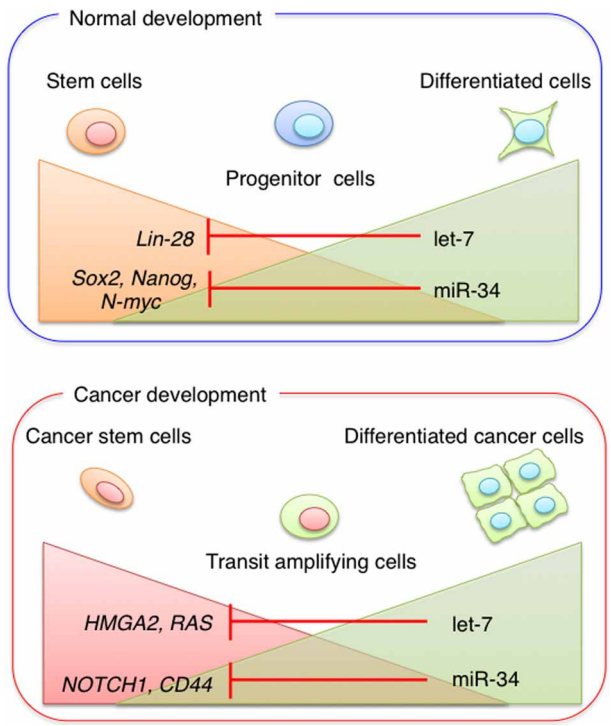

FIGURE 2 | miRNA in stem cells and cancer stem cells. Stem cell-specific miRNAs play important roles in tumor initiation and development. During normal development, pluripotent stem cells become more restricted to specific cell lineages. Progenitor cells are committed to generating different cell types, whereas fully differentiated cells have a low potential for self-renewal. The expression levels of miR-34 and let-7 family members increase during differentiation. During cancer development, CSC properties are regulated by the balance between miRNA expression and the expression of miRNA target genes.

CSCs

Accumulating lines of evidence suggest that CSCs share a variety of biological properties with normal somatic stem cells such as the capacity for self-renewal, the propagation of differentiated progenitors, and the expression of specific stem cell genes (Colmont et al., 2012). However, CSCs differ from normal stem cells in their chemoresistance and tumorigenic and metastatic activities (Colmont et al., 2012 and **Table 1**). In addition, recently glycosylation patterns are found to be different between normal stem cells and CSCs (Karsten and Goletz, 2013). The CSC theory is generally accepted in the field of cancer research, not only in basic research but also with regard to cancer drug discovery.

Normal stem cells and CSCs act via common signaling pathways that regulate self-renewal activity, including Wnt, Notch,

Table 1 | Representative cell surface markers for human CSCs.

Cancer type	CSC marker	References
AML	$CD34^+/CD38^-$	Bonnet and Dick, 1997
Breast	$CD44^+/CD24^{-/low}$	Al-Hajj et al., 2003
	ALDH1	Ginestier et al., 2007
Glioma	CD133	Singh et al., 2003, 2004
Colon	CD133	O'brien et al., 2007; Ricci-Vitiani et al., 2007
	CD44/EpCAM/CD166	Dalerba et al., 2007
Metastatic Colon	$CD133^+/CD26^+$	Pang et al., 2010
Melanoma	CD20	Fang et al., 2005
	CD271	Boiko et al., 2010
Pancreatic	ESA/CD44/CD24	Hermann et al., 2007
Metastatic Pancreatic	CD133/CXCR4	Li et al., 2007a
Prostate	CD44/a2β1/CD133	Collins et al., 2005
Lung	CD133	Eramo et al., 2008
Hepatic	EpCAM/AFP	Yamashita et al., 2010
Gastric	CD44	Takaishi et al., 2009

AML, acute myelogenous leukemia; ALDH, aldehyde dehydrogenase; EpCAM, epithelial cell adhesion molecule; CXCR4, CXC chemokine receptor 4; AFP, alpha-fetoprotein.

and Sonic Hedgehog, and dysregulation of these pathways plays a role in tumor initiation and development (Reya et al., 2001). Jamieson et al. showed that aberrations in the Wnt/β-catenin pathway enhance self-renewal activity during leukemia stem cell propagation (Jamieson et al., 2004). Korkaya et al. reported that the Wnt/β-catenin pathway is involved in the regulation of normal and malignant mammary stem/progenitor cell populations (Korkaya et al., 2009). Several studies have shown that the Notch pathway is activated in breast, glioblastoma, and colon CSCs (Hoey et al., 2009; Taketo, 2011). Alterations in Hedgehog signaling have been reported in colon, breast, and glioblastoma CSCs (Liu et al., 2006; Varnat et al., 2009; Takezaki et al., 2011).

The development of fluorescent antibodies, flow cytometry, and cell sorting techniques enabled the identification of cell populations possessing CSC properties. Furthermore, the development of severely immunodeficient mouse strains facilitated the evaluation of tumor formation ability. These methods have enabled the identification and isolation of CSCs from various cancers (Bonnet and Dick, 1997; Al-Hajj et al., 2003; Collins et al., 2005; Fang et al., 2005; Ginestier et al., 2007; Hermann et al., 2007; Li et al., 2007a; Eramo et al., 2008; Takaishi et al., 2009; Boiko et al., 2010; Pang et al., 2010; Yamashita et al., 2010) (**Table 1**). In this review, we discuss the major findings of recent studies highlighting the roles of certain "CSC-specific" miRNAs in representative cancer types (**Table 2**). From these discussions, we present an emerging theme that several miRNAs may exert a functional role in the regulation of the key biological properties of CSCs.

LEUKEMIA STEM CELLS

Through an integrated approach that combined miRNA expression analysis and bioinformatic prediction of mRNA targets, distinct miRNA signatures were shown to fine-tune each step of hematopoiesis, including the reconstitution potential of hematopoietic stem cells (Arnold et al., 2011). The miR-17-92 cluster functions as an oncogenic miRNA by enhancing the formation of Myc-driven B-cell lymphomas in a mouse model (He et al., 2005). Single miRNAs function as oncogenes. The overexpression of miR-155 in early B-cells leads to polyclonal expansion of the pro-B-cell compartment (Costinean et al., 2006), and retroviral expression of miR-155 in immature mouse hematopoietic cells resulted in the expansion of granulocyte/monocyte populations displaying pathological features characteristic of myeloid neoplasia without progression to acute myeloid leukemia (AML) (O'connell et al., 2008). Recently, dysregulation of single miRNAs was shown to contribute to hematological malignancies, including AML and myelodysplastic syndrome (Han et al., 2010; Song et al., 2013a). Han et al. reported that miR-29a regulates early hematopoiesis and induces AML by converting myeloid progenitors into self-renewing leukemia stem cells via targeting several tumor suppressors and cell cycle regulators (Han et al., 2010). miR-22-induced inhibition of the ten-eleven-translocation gene 2 (*TET2*) tumor suppressor increased the methylation of TET2 target genes, such as *Aim2*, *Hal*, *Igbt2*, and *Sp140*, and resulted in positive effects on hematopoietic stem cell self-renewal and transformation. This has led to the suggestion that mir-22 is associated with myelodysplastic syndrome and hematological malignancies (Song et al., 2013a).

BREAST CSCs

The first solid tumor CSCs were identified in and isolated from breast tumors in 2003 (Al-Hajj et al., 2003). Al-Hajj et al. described a $CD44^+/CD24^{-/low}$ cell population that had a markedly high tumor-initiating capacity. In 2007, Yu et al. identified let-7 as a master regulator of breast CSC properties (Yu et al., 2007a). In breast CSCs, reduced let-7 expression controls self-renewal and differentiation through *RAS* and *HMGA2*, respectively (**Figure 2**). Since HMGA2 plays a role in the control of differentiation and proliferation of both human and mouse ESCs (Li et al., 2007b), these findings also suggest that let-7 is involved in the growth and differentiation of ESCs beyond tumorigenesis.

Epithelial-to-mesenchymal transition (EMT) is an evolutionarily conserved process that occurs during embryonic development in many species of mammals (Liu et al., 2006). Since the EMT program is often activated during tumor invasion and metastasis, the genetic controls and biochemical mechanisms underlying the acquisition of invasiveness and the subsequent systemic spread of cancer cells have been areas of intensive research. The EMT phenotype is characterized by the downregulation of epithelial markers such as E-cadherin, the expression of mesenchymal markers such as N-cadherin and vimentin, the loss of cell-cell contact and cell polarity, and the acquisition of cell invasive capabilities. Mani et al. reported that EMT is also associated with the acquisition of CSC properties (Mani et al., 2008). A $CD44^+/CD24^{-/low}$ cell population purified from cancer tissues shows the features of an EMT phenotype, and human cancer cells induced to undergo EMT exhibit a $CD44^+/CD24^{-/low}$ antigen phenotype and high tumorigenicity.

Recently, two studies reported the clinical relevance of CSCs in breast cancer specimens (Giordano et al., 2013; Yu et al., 2013). In early breast cancer patients, the presence of $CD44^+/CD24^{-/low}$ cells in bone marrow was indicative of a poor prognosis (Giordano et al., 2013). Circulating tumor cells (CTCs) in breast cancer patients also showed the EMT phenotype (Yu et al., 2013). Progressive disease patients undergoing therapy had a higher number of mesenchymal marker positive CTCs than epithelial marker positive CTCs. These results suggest that the CSC phenotype is clinically important not only as a therapeutic target but also as a potential biomarker for the prognostic evaluation of patients undergoing cancer treatment.

A molecular link between EMT and the miR-200 family is provided by the zinc-finger E-box-binding homeobox protein encoding genes (*ZEB1/ZEB2*) (Gregory et al., 2008; Park et al., 2008). The miR-200 family consists of five members that are classified into two clusters: miR−200a, miR−200b, and miR−429 on human chromosome 1; and miR−200c and miR−141 on human chromosome 12 (Gregory et al., 2008). Expression of the miR-200 family strongly inhibits the EMT phenotype induced by TGF-β, and a reciprocal feedback loop between the miR-200 family and the ZEB family of transcription factors tightly regulates both EMT and mesenchymal-to-epithelial transition (Burk et al., 2008). MiR-200 family members are downregulated in normal human and mouse mammary stem cells and breast CSCs, and miR-200c inhibits the formation of mammary ducts from mammary stem cells and tumor formation from breast

Table 2 | The regulatory roles of miRNAs in CSCs.

Cancer Type	miRNA	Target gene	Role of miRNA in CSC properties	References
Leukemia (AML and MDS)	miR-22	*TET2*	Promotion of self-renewal	Song et al., 2013a
Breast	Let-7	*RAS and HMGA2*	Inhibition of self-renewal and de-differentiation	Yu et al., 2007a
	miR-200 family	*ZEB1/ZEB2* *BMI-1* *SUZ12*	Inhibition of EMT Inhibition of self-renewal Inhibition of mammosphere formation	Gregory et al., 2008 Shimono et al., 2009 Iliopoulos et al., 2010
	miR-22	*TET* family *(TET1 -3)*	Suppression of miR-200 family expression	Song et al., 2013b
Brain	miR-9/9*, miR-17	*CAMTA1*	Promotion of $CD133^+$ cell proliferation	Schraivogel et al., 2011
	miR-128	*BMI-1*	Inhibition of self-renewal	Godlewski et al., 2008
	miR-199b-5p	*HES1*	Reduction of the $CD133^+$ cell fraction	Garzia et al., 2009
Colon	miR-193	*PLAU and K-RAS*	Inhibition of tumorigenicity and invasiveness	Iliopoulos et al., 2011
	miR-451	*MIF and COX-2*	Inhibition of self-renewal and tumorigenicity	Bitarte et al., 2011
	miR-34a	*NOTCH 1*	Suppression of asymmetric cell division	Bu et al., 2013
Prostate	miR-34a miR-320	*CD44* β-*catenin*	Inhibition of self-renewal and metastasis Inhibition of Wnt/β-catenin pathway	Liu et al., 2011 Hsieh et al., 2013

AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome.

CSCs (Shimono et al., 2009). Members of the miR-200 family also modulate the self-renewal ability of CSCs by targeting B-lymphoma Mo-MLV insertion region 1 homolog (*BMI-1*) and *SUZ12*, a subunit of a polycomb repressor complex (Iliopoulos et al., 2010). BMI-1 regulates the self-renewal and differentiation of several types of stem cells, including hematopoietic, brain, and mammary stem cells (Molofsky et al., 2003; Park et al., 2003; Pietersen et al., 2008). Therefore, modulation of the activity of the miR-200 family using conventional therapy could be a promising approach to improve the effectiveness of breast cancer treatments.

Normal human and mouse mammary stem cells can be isolated and characterized on the basis of their aldehyde dehydrogenase (ALDH) activities (Ginestier et al., 2007). Using ALDH activity, Ibara et al. determined that miR-205 and miR-22 were highly expressed in mouse mammary progenitor cells (Ibarra et al., 2007). MiR-22 was recently shown to be an epigenetic modifier that promotes stemness and metastasis in breast cancer by directly targeting enzymes in the TET family, which regulate DNA demethylation (Song et al., 2013b). The TET family is involved in the demethylation of the miR-200 promoter, and miR-22 promotes CSC properties such as EMT and a metastatic phenotype through the suppression of the miR-200 family. This provides the first evidence that chromatin-remodeling systems with opposing effects on cell fate (self-renewal vs. differentiation) are regulated by opposing sets of miRNAs.

BRAIN CSCs

The pentaspan membrane glycoprotein CD133, also known as Prominin-1, was first identified as a marker of hematopoietic stem cells and progenitor cells, and was subsequently used to detect malignancies (Miraglia et al., 1997; Yin et al., 1997). In solid cancers, CD133 was first used to identify CSCs in different types of human brain tumors including glioblastoma, medulloblastoma, and ependymomas (Singh et al., 2003, 2004; Yu et al., 2010). In these studies, patient tumor cells were separated based on the expression of CD133. The $CD133^+$ cell population is highly tumorigenic *in vivo*, whereas $CD133^-$ cells do not form tumors even at high numbers (Singh et al., 2003, 2004; Yu et al., 2010). $CD133^+$ cells are also resistant to radiation and chemotherapy. These findings led to the hypothesis that glioblastomas are maintained by CSCs, and that this treatment-resistant subpopulation is a promising target for effective therapies. CD133 has been instrumental for the identification of CSCs in colorectal (Ricci-Vitiani et al., 2007) and pancreatic (Hermann et al., 2007) carcinomas. CD133 itself is a marker of normal neural stem cells in both humans (Uchida et al., 2000) and mice (Lee et al., 2005).

In cancer cells, the deacetylase HDAC6 directly interacts with and regulates the intracellular localization of CD133 (Mak et al., 2012). CD133 forms a stable protein complex with HDAC6 and β-catenin, which leads to the activation of β-catenin signaling targets in different types of cancer. CD133 is also associated with phosphoinositide 3-kinase (PI3K) 85 kDa regulatory subunit (p85) in glioma stem cells (GSCs) (Wei et al., 2013). The PI3K pathway is a key regulator of tumorigenesis in glioblastoma and other cancers (Godlewski et al., 2010). Therefore, activation of the PI3K/Akt pathway by the physical interaction between CD133 and p85 promotes tumorigenicity in GSCs. The function of CD133 in brain tumors should be fully characterized in the near future, which may shed light on the role of CD133 as a functional marker of GSCs.

Schraivogel et al. reported that miR-9, miR-9* (miR-9/9*), miR-17, and miR-106b are highly abundant in the $CD133^+$ cell population in glioblastoma cell lines. Among the upregulated miRNAs in the $CD133^+$ cell population, inhibition of miR-9/9* or miR-17 leads to reduced neurosphere formation and stimulates cell differentiation. Functional analysis of these miRNAs showed that miR-9/9* and miR-17 target calmodulin-binding transcription activator 1 (*CAMTA1*), a putative transcription factor of the anti-proliferative cardiac hormone natriuretic peptide A (*NPPA*). Clinical studies also demonstrated that *CAMTA1* and *NPPA* expression is correlated with patient survival. These findings could provide a basis for the design of novel treatment strategies for glioblastoma (Schraivogel et al., 2011).

MiR-124 and miR-128 are the most highly expressed miRNAs in the adult brain and are preferentially expressed in neurons (Smirnova et al., 2005). Patients with high-grade glioma show significant downregulation of miR-128 expression. Functional analyses showed that miR-128 expression inhibits glioma cell proliferation *in vitro* and glioma xenograft growth *in vivo* (Godlewski et al., 2008). In addition, miR-128 specifically inhibits the self-renewal capacity of GSCs by directly targeting *BMI-1*, a polycomb family transcriptional repressor required for postnatal maintenance of neural stem cells in the peripheral and central nervous system (Molofsky et al., 2003). Since BMI-1 maintains neural stem cells in an undifferentiated self-renewing state, the regulation of *BMI-1* by miR-128 may contribute to normal stem cell regulation.

Another study showed that miR-199b-5p downregulation was associated with metastatic spread in medulloblastoma. In medulloblastoma cells, miR-199b-5p directly targets *HES1*, a transcription factor of the Notch signaling pathway (Garzia et al., 2009). During brain development, Notch functions as a critical regulator of cell fate, by which gliogenesis can only occur when Notch signaling specifically represses the neuronal pathway in progenitor cells (Karamboulas and Ailles, 2013). MiR-199b-5p blocks Notch signaling, inhibiting the self-renewal capacity of medulloblastoma cells by reducing the $CD133^+$ subpopulation (Garzia et al., 2009). Recently, miR-34a was shown to regulate Notch signaling by targeting *Notch-1* and *Notch-2* in medulloblastoma cells (Li et al., 2009). Therefore, miR-199b-5p and miR-34a are important for the self-renewal potential of GSCs via the Notch signaling pathway.

COLON CSCs

CD133 was initially used to identify and isolate colon CSCs (O'brien et al., 2007; Ricci-Vitiani et al., 2007), which was followed by the identification of CD44, epithelial surface antigen (EpCAM), and CD166 as alternative colon CSC markers (Dalerba et al., 2007). CD166 is a mesenchymal stem cell marker whose expression is correlated with poor prognosis in colon cancer patients (Weichert et al., 2004). Compared to $CD44^-/EpCAM^{low}$ cells, $CD44^+/EpCAM^{high}$ cells from primary tumors show high tumorigenic activity in NOD/SCID mice. Moreover, $CD166^+$ cells in the $CD44^+/EpCAM^{high}$ cell fraction contribute to the tumorigenic activity of colon CSCs. In addition to CD133, CD44, EpCAM, and CD166, the expression of leucine-rich repeat-containing G-protein-coupled receptor 5 (Lgr5) varies among colorectal cancer (CRC) cases and is significantly correlated with lymphatic and vascular invasion, lymph node metastasis, and drug resistance (Vermeulen et al., 2008; Merlos-Suarez et al., 2011; Kobayashi et al., 2012).

Iliopoulos et al. reported that the expression of miR-193a is inversely correlated with *K-RAS* and plasminogen activator urokinase (*PLAU*) expression in human colon adenocarcinomas, and that miR-193 expression inhibits tumorigenicity and invasiveness by directly targeting *K-RAS* and *PLAU*, respectively (Iliopoulos et al., 2011). MiR-451 is another regulator of CSC properties such as self-renewal, tumorigenicity, and drug resistance. In spheroid cell culture, downregulation of miR-451 induces the upregulation of macrophage migration inhibitory factor (MIF) and COX-2, resulting in the acquisition of self-renewal and tumorigenic properties (Bitarte et al., 2011). MIF and Cox-2 are involved in the activation of the Wnt pathway, which is functionally essential for the maintenance of colon CSCs (Vermeulen et al., 2010), suggesting that miR-451 could regulate the properties of colon CSCs by suppressing the Wnt pathway.

Notch signaling is frequently activated in CRCs, and is dysregulated directly by epigenetic and genetic changes and indirectly by synergistic interactions with the Wnt pathway, which is also activated in CRC (Taketo, 2011). Notch signaling promotes the self-renewal activity of intestine and colon stem cells (Taketo, 2011). Therefore, colon CSCs in CRC are thought to arise from, or at least share common properties with, normal colon stem cells (Clevers, 2011; O'brien et al., 2012). Bu et al. reported that miR-34a determines whether colon CSCs undergo symmetric or asymmetric division, and that inhibition of asymmetric cell division suppresses tumorigenicity (Bu et al., 2013). MiR-34a inhibits Notch signaling by directly targeting Notch receptors (Li et al., 2009), suggesting that the upregulation of miR-34a weakens Notch signaling and promotes the generation of daughter cells (non-CSCs), whereas low miR-34a levels promote Notch signaling and lead to the maintenance of CSCs. This study also demonstrated that the expression level of miR-34a correlates more closely with the differentiation of daughter cells than the presence of Numb, which also suppresses Notch signaling by promoting the degradation of membrane-bound Notch and its intracellular domain (Bu et al., 2013).

PROSTATE CSCs

In prostate cancer (PCa), $\alpha_2\beta_1$ integrin, CD133, and CD44 were initially used to identify and isolate CSCs (Collins et al., 2005; Patrawala et al., 2006, 2007). Patrawala et al. reported that $CD44^+$ PCa cells have higher proliferative, tumorigenic, and metastatic potentials than $CD44^-$ PCa cells (Patrawala et al., 2006), and showed that androgen receptor (AR)-negative $CD44^+$ PCa cells differentiate into AR-positive $CD44^-$ PCa cells. Consistent with this report, prostate-specific antigen (PSA)-negative or -low PCa cells that are resistant to androgen ablation have a highly tumorigenic phenotype (Qin et al., 2012). In addition, $PSA^{-/low}$ PCa cells generate PSA^+ PCa cells through asymmetric cell division, and highly tumorigenic $PSA^{-/low}$ PCa cells are characterized by an $ALDH^+/CD44^+/\alpha_2\beta_1$ integrin$^+$ phenotype (Qin et al., 2012).

Liu et al. reported that miR-34a is downregulated in $CD44^+$ PCa cells purified from xenografts and primary tumors, and

that miR-34a directly regulates the expression of *CD44* at the post-transcriptional level by binding to its 3′UTR (Liu et al., 2011). Expression of miR-34a in $CD44^+$ PCa cells inhibits tumor migration and metastasis in a xenograft model (Liu et al., 2011), and miR-34a inhibits Notch and AR signaling in PCa cells (Li et al., 2009; Kashat et al., 2012), suggesting that miR-34a suppresses the self-renewal activity of CSCs in PCa cells.

Another miRNA that regulates CSC properties is miR-320, which acts by directly targeting β-catenin in PCa cells (Hsieh et al., 2013). miR-320 and β-catenin expression is inversely correlated in $CD44^+$ PCa cells. Furthermore, gene expression profiling of miR-320-overexpressing PCa cells showed a significant decrease in downstream target genes of the Wnt/β-catenin pathway and CSC markers (Hsieh et al., 2013).

THERAPEUTIC APPROACHES TO TARGET CSCs

The development of therapies against CSCs has resulted in the establishment of a new generation of cancer therapeutics, which is particularly important in the treatment of intractable cancers. Since CSCs are molecularly distinct from non-CSCs and bulk tumor cells, a high-throughput screening approach was used to identify small compounds that eliminate or reduce levels of CSCs (Gupta et al., 2009; Sachlos et al., 2012). Gupta et al. identified salinomycin as a selective inhibitor of breast CSCs (Gupta et al., 2009) by screening a library of 16,000 natural and commercial chemical compounds in a search for small compounds capable of killing breast CSCs. Although the precise molecular mechanisms underlying the elimination of CSCs by salinomycin are not fully understood, several studies have improved our understanding of the mechanisms and pharmacological action of salinomycin in human CSCs (Fuchs et al., 2010; Lu et al., 2011; Tang et al., 2011). Systemic salinomycin therapy induces a marked regression of subcutaneous thoracal metastases of breast cancer, and combination therapy of salinomycin with erlotinib resulted in significant tumor regression in metastatic squamous cell carcinoma (Naujokat and Steinhart, 2012).

High-throughput screening using neoplastic and normal human pluripotent stem cells (hPSC) showed that among 590 compounds, only thioridazine significantly promoted differentiation of neoplastic hPSCs but not of normal hPSCs (Sachlos et al., 2012). Thioridazine acts through dopamine receptors (dopamine receptor1-5) (Seeman and Lee, 1975), indicating that its selective interference with human CSCs is mediated by dopamine receptor antagonism.

The development of therapies against CSCs is challenging because both bulk cancer cells and CSCs must be eliminated. As CSCs are molecularly distinct from bulk tumor cells, they can be targeted by exploiting their molecular differences as described above (**Tables 1**, **2**). One of the most promising approaches is the cell based delivery of miRNAs or miRNA inhibitors. Several studies demonstrated that miRNAs are secreted through "exosomes," which are small endosome-derived vesicles (30–100 nm) secreted from different cell types, such as dendritic cells, hepatocyte, and tumor cells (Mittelbrunn et al., 2011; Luga et al., 2012; Ramakrishnaiah et al., 2013). The exosome secreted from mesenchymal stem cells (MSC) is selectively transferred to the glioblastoma multiforme (GBM) (Munoz et al., 2013). Since miR-9 is involved in the upregulation of p-glycoprotein, Munoz et al. developed an MSC derived exosome containing anti-miR-9 that efficiently suppressed p-glycoprotein expression in the temozolomide-resistant GBM.

The glycosylation pattern of CSC markers on CSCs is different from normal stem cells (Karsten and Goletz, 2013). Some CSC markers such as CD44 and CD133 are also expressed in normal stem and progenitor cells (Karsten and Goletz, 2013), which might have negative implications for the development of CSC-targeted delivery. This problem could be addressed by the development of liposomes or nanoparticles conjugated to antibodies against CSC specific glycans that permit the selective delivery of CSC suppressive miRNAs or small molecules.

Recent studies have shown that several dietary compounds can directly or indirectly affect the properties of CSCs (Li et al., 2011). Therefore, natural dietary compounds have received increasing attention in cancer chemoprevention, and several natural compounds that induce the elimination or differentiation of breast CSCs have been identified (Kakarala et al., 2010; Li et al., 2010; Hagiwara et al., 2012). Resveratrol is a non-toxic natural product that is found in grapes, berries, peanuts and red wine (Aziz et al., 2003). Nowadays, resveratrol is widely consumed as a nutritional supplement (Prasad, 2012), and its multifaceted biological effects include anti-mutagenic and anti-cancer properties (Prasad, 2012; Patel et al., 2013). Hagiwara et al. found that resveratrol enhances miRNA functions through the upregulation of Ago2 expression, which leads to the suppression of CSC properties (Hagiwara et al., 2012). These results suggest that the identification of non-toxic natural compounds capable of suppressing the properties of CSCs through the regulation of miRNA expression is a promising approach to support conventional chemotherapy.

CONCLUSIONS

Accumulating lines of evidence have shown that the heterogeneity and plasticity of cancer cells is reflected in the transition from a non-CSC to a CSC phenotype. Therefore, clinical oncologists and cancer researchers need to determine which cancer cells have the potential to contribute to tumor initiation and progression, including therapeutic resistance and metastasis. Several studies reviewed here have shown that miRNAs can function as tumor suppressors or oncogenes and play important roles in various aspects of CSC properties. In this regard, miRNAs are considered to be functional markers of CSCs. Therefore, a more detailed understanding of the function of miRNAs in CSC biology may improve cancer treatments and possibly lead to the clinical application of miRNAs in cancer diagnosis, treatment, and prognosis.

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Disruption of the expression and function of microRNAs in lung cancer as a result of epigenetic changes

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Two decades have passed since the discovery of microRNA (miRNA), which determines cell fate in nematodes. About one decade ago, the conservation of miRNA in humans was also discovered. At present, the loss of certain miRNAs and the overexpression of miRNAs have been demonstrated in many types of diseases, especially cancer. A key miRNA in lung cancer was reported soon after the initial discovery of a tumor-suppressive miRNA in a hematological malignancy. Various causes of miRNA disruption are known, including deletions, mutations, and epigenetic suppression as well as coding genes. The recent accumulation of knowledge regarding epigenetic transcriptional suppression has revealed the suppression of several miRNAs in lung cancer in response to epigenetic changes, such as H3K9 methylation prior to DNA methylation and H3K27 methylation independent of DNA methylation. In this review, recent knowledge of miRNA disruption in lung cancer as a result of epigenetic changes is discussed. Additionally, emerging cancer-specific changes in RNA editing and their impact on miRNA function are described.

Keywords: microRNA, lung cancer, DNA methylation, histone modification, RNA editing

MICRORNAs AND CANCER: A HISTORICAL PERSPECTIVE

MicroRNAs (miRNAs) are small non-coding RNA molecules (approximately 22 nucleotides) that function as versatile regulators of gene expression. miRNAs negatively regulate the expression of thousands of genes through the destabilization and/or translational suppression of mRNAs by binding to complementary sequences in the 3′ untranslated regions (3′UTRs) of target mRNAs (Lee et al., 1993; Wightman et al., 1993).

The first miRNA to be discovered, *lin-4*, was determined to be an essential regulator of development in the nematode *Caenorhabditis elegans* (Lee et al., 1993; Wightman et al., 1993). This short non-coding RNA was considered to be a peculiar constituent specific to worms. Seven years passed before a second miRNA, *let-7*, was discovered in nematodes (Reinhart et al., 2000). Shortly thereafter, *let-7* was found to be broadly conserved across many species, including humans (Pasquinelli et al., 2000). In 2001, a large number of such genes were identified, and the term "microRNA" was coined (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Currently, more than 2,000 mature miRNAs have been documented in the miRNA registry (Sanger miRBase release 20; http://www.mirbase.org).

MicroRNAs are involved in many biological processes such as cell cycle control, cell differentiation, and apoptosis. Alterations in miRNA expression have been increasingly recognized as playing important roles in the pathogenesis of human cancers. For example, the first tumor-suppressive miRNAs *mir-15* and *mir-16* located at 13q14 are frequently deleted and downregulated in chronic lymphocytic leukemia (Calin et al., 2002). In lung cancer, a reduction in *let-7* expression is significantly associated with a shorter postoperative survival (Takamizawa et al., 2004). The *let-7* miRNAs target important oncogenes such as the *Ras* family (Johnson et al., 2005) and *HMGA2* (Mayr et al., 2007). The *mir-17-92* miRNA cluster, which was the first oncogenic miRNA to be reported, is amplified and over expressed in B cell lymphoma (He et al., 2005). Moreover, the *mir-17-92* miRNA cluster is also amplified and overexpressed in small-cell lung cancer (SCLC) and enhances the proliferation of cancer cells (Hayashita et al., 2005).

MicroRNAs can be used as biomarkers for the diagnosis and prognosis of malignancies. In general, miRNA expression is downregulated in tumors, compared with normal tissues, and analyzes of the expression of 217 miRNAs in various human cancers reflect the developmental lineage and differentiation of the tumor (Lu et al., 2005). Furthermore, certain miRNAs can aid in classifying the histological subtype (adenocarcinoma or squamous cell carcinoma) of lung cancer (Bishop et al., 2010). The miRNA signature can also predict the survival and relapse of patients with lung cancer (Yu et al., 2008).

Despite growing evidence of the involvement of miRNAs in human carcinogenesis, limited information is available regarding how miRNA expressions are deregulated in cancer. In this article, we review the mechanisms responsible for the changes in miRNA expression in lung cancer, focusing particularly on epigenetic mechanisms, such as DNA methylation and histone modifications.

MECHANISMS OF DEREGULATED miRNA EXPRESSION IN CANCER

In animals, miRNAs are generally transcribed by RNA polymerase II (Lee et al., 2004) to form primary transcripts (pri-miRNAs). Pri-miRNAs form hairpin structures in the nucleus and are processed by the Drosha/DGCR8 complex to form approximately 60 nt precursor miRNAs (pre-miRNAs; Gregory et al., 2004).

Pre-miRNAs are transported to the cytoplasm through the RAN GTP-dependent transporter exportin-5 (Lund et al., 2004) and are cleaved by Dicer into mature miRNAs (Hutvagner et al., 2001; **Figure 1**).

miRNA are frequently located at fragile sites as well as minimal regions of loss of heterozygosity, minimal regions of amplification, or common breakpoint regions in cancer (Calin et al., 2004). In addition to such genomic changes, any alteration in the miRNA biogenesis pathway described above can affect miRNA expression in cancer. The currently known mechanisms responsible for changes in miRNA expression in cancer include genomic deletions or amplifications, chromosomal translocations, epigenetic silencing by DNA methylation, and impairments of the miRNA biogenesis pathway, such as the frameshift mutation of *exportin-5* (Melo et al., 2010), the downregulation of Dicer (Karube et al., 2005; Merritt et al., 2008), and the frameshift mutation of *TARBP2* (Melo et al., 2009).

As mentioned above, *mir-15* and *mir-16*, located at 13q14, are deleted in more than half of all cases of chronic lymphocytic leukemia (Calin et al., 2002), and amplification of the *mir-17-92* miRNA cluster located at 13q31 is observed in B cell lymphoma and SCLC (Hayashita et al., 2005; He et al., 2005). In addition, *mir-125b*-1 is a target of the chromosomal translocation *t*(11,14) in B-cell precursor acute lymphoblastic leukemia, and this translocation results in *mir-125b* overexpression that is controlled by an immunoglobulin heavy-chain gene regulatory element (Bousquet et al., 2008; Chapiro et al., 2010). However, with regard to lung cancer, no chromosomal translocations involving miRNAs have been previously reported.

Kumar et al. (2007) reported that the impairment of the miRNA biogenesis pathway in cancer results in the global downregulation of miRNA and the enhancement of cellular transformation and tumorigenesis. They demonstrated that the knockdown of DGCR8, Drosha, or Dicer in cancer cells resulted in a pronounced transformed phenotype and that the conditional deletion of *Dicer* enhanced tumor development in a *K-ras*-induced mouse model of lung cancer. Importantly, a reduction in Dicer expression is associated with a poor prognosis in patients with lung cancer (Karube et al., 2005) and ovarian cancer (Merritt et al., 2008). Interestingly, a germline mutation of *Dicer1* has been discovered in pleuropulmonary blastoma, a rare pediatric lung tumor (Hill et al., 2009). These findings clearly demonstrate that the miRNA biogenesis pathway plays a crucial role in normal lung development and lung carcinogenesis. Frameshift mutations of *exportin-5* and *TARBP2* have been reported in colorectal cancer with microsatellite instability, but not in lung cancer (Melo et al., 2009, 2010).

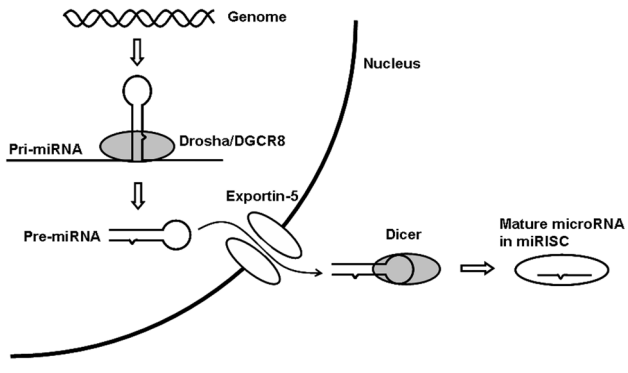

FIGURE 1 | miRNA biogenesis pathway. miRISC, microRNA-induced silencing complex.

Epigenetic changes in cancer genomes, such as DNA methylation and histone modifications, cause the silencing of tumor suppressor genes and contribute to human carcinogenesis (Jones and Takai, 2001; Jones and Baylin, 2002; Herman and Baylin, 2003). Recently, DNA methylation in cancerous tissue has been shown to silence miRNAs in cancer (Saito et al., 2006; Lujambio et al., 2007). Saito et al. reported that the expression of *mir-127*, which is embedded in a CpG island, was induced by treatment with the chromatin-modifying drugs 5-aza-2'-deoxycytidine and 4-phenylbutyric acid in a bladder cancer cell line. Lujambio et al. analyzed the miRNA expression profile of a colon cancer cell line, which was genetically deficient for the DNA methyltransferase (DNMT) enzymes *DNMT1* and *DNMT3b*, and identified the epigenetic silencing of *mir-124a* in various types of cancer, including colon, breast, and lung cancers as well as leukemia and lymphoma. As the epigenetic silencing of key tumor suppressor genes, such as *APC* and *p16INK4A*, is a common event in lung carcinogenesis (Takai et al., 2001; Sano et al., 2007; Brock et al., 2008; Kusakabe et al., 2010) and miRNA expression is altered in lung cancer (Yanaihara et al., 2006), some miRNAs are thought to be silenced by DNA methylation or histone modification in lung cancer. In fact, growing evidence demonstrates that epigenetic changes contribute to miRNA silencing in lung cancer (Liu et al., 2013).

DNA METHYLATION AND miRNA EXPRESSION

The earliest papers on miRNA expression profiling in lung cancer were published in 2006 (Volinia et al., 2006; Yanaihara et al., 2006). These studies used miRNA microarrays and found that a high level of *mir-155* expression and a low level of *let-7a-2* expression were significantly correlated with a poor survival of lung cancer patients. The number of miRNA profiling studies in lung cancer has grown rapidly in recent years, and these studies have led to the discovery of the role of miRNAs in lung carcinogenesis and their potential as diagnostic, prognostic, or predictive markers (Rothschild, 2013). Vosa et al. performed a meta-analysis of 20 published miRNA expression profiling studies in lung cancer and identified a meta-signature of seven up-regulated (*mir-21, mir-210, mir-182, mir-31, mir-200b, mir-205, and mir-183*) and eight down-regulated (*mir-126-3p, mir-30a, mir-30d, mir-486-5p, mir-451a, mir-126-5p, mir-143, and mir-145*) miRNAs (Vosa et al., 2013). Guan et al. (2012) also reported a meta-analysis of 14 published miRNA expression profiling studies and their results agreed well with those published by Vosa et al.

One approach to identifying epigenetically silenced miRNAs is to analyze the miRNA expression profile of cancer cells before and after treatment with chromatin-modifying drugs. Lujambio et al. analyzed the miRNA expression profile of three metastatic cancer cell lines with and without the DNMT inhibitor 5-aza-2'-deoxycytidine and reported that the DNA methylation of three miRNAs (*mir-9*, *mir-34b/c*, and *mir-148a*) was associated

with the metastasis of human cancers including lung cancer (Lujambio et al., 2008). Heller et al. analyzed the miRNA expression profile changes in A549 lung cancer cells treated with 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor tricostatin A and identified *mir-9-3* and *mir-193a* as targets for DNA methylation in non-small cell lung cancer (NSCLC; Heller et al., 2012). Our research team analyzed the expressions of 55 *in silico* selected candidate miRNAs treated with or without 5-aza-2'-deoxycytidine and found that *mir-34b/c* and *mir-126* are silenced by DNA methylation in NSCLC (Watanabe et al., 2012). We also revealed that the DNA methylation of *mir-9*-3, *-124-2*, and *-124-3* was individually associated with an advanced T factor, and that the methylation of multiple miRNA loci was associated with a poor prognosis (Kitano et al., 2011). The correlation between miRNA methylation and the T factor suggested that the DNA methylation of these miRNA loci accumulates during tumor progression. A list of miRNAs that are silenced by DNA methylation in lung cancer is shown in **Table 1**.

The *mir-34* family is comprised of three miRNAs (*mir-34a*, *mir-34b*, and *mir-34c*) that are derived from two transcripts (*mir-34a* on chromosome 1 and *mir-34b/c* on chromosome 11). In mice, *mir-34a* is ubiquitously expressed, with the highest expression being in the brain, whereas *mir-34b/c* is mainly expressed in the lung (Bommer et al., 2007). The *mir-34* genes induce cell cycle arrest, cellular senescence, and apoptosis when ectopically expressed (Bommer et al., 2007; He et al., 2007; Welch et al., 2007) through the downregulation of multiple target genes such as *Bcl-2*, *Cyclin D1*, *Cyclin E2*, *CDK4*, *CDK6*, *c-Myc*, and *c-Met* (Hermeking, 2010). Moreover, *mir-34*s have been identified as direct targets of the p53 transcription factor (Bommer et al., 2007; Corney et al., 2007; He et al., 2007), and their expression is induced in response to DNA damage or oncogenic stress. These results indicate that *mir-34*s play a critical role in the tumor-suppressive program governed by p53. Interestingly, the chromosomal locus 1p36, where *mir-34*a is located, has been proposed to harbor a tumor suppressor gene, since a homozygous deletion at this locus has been reported in neuroblastoma, and *mir-34*a has been identified as a candidate tumor suppressor at this locus (Cole et al., 2008).

In lung cancer, *mir-34a* and *mir-34b/c* are targets of epigenetic silencing by DNA methylation (Lodygin et al., 2008; Gallardo et al., 2009; Wang et al., 2011; Watanabe et al., 2012). In primary NSCLC, a low *mir-34a* expression level is significantly associated with a high probability of relapse after surgery (Gallardo et al., 2009). We previously reported that *mir-34b/c* is methylated in 41% of primary NSCLC cases and that *mir-34b/c* methylation is associated with lymphatic invasion (Watanabe et al., 2012). The DNA methylation of *mir-34b/c* is associated with a poorer prognosis in patients with NSCLC (Wang et al., 2011). In addition, the *mir-34*s are silenced by DNA methylation in SCLC (Tanaka et al., 2012). In primary SCLC, *mir-34a* and *mir-34b/c* were methylated in 15% and 67% of the cases, respectively. The CpG island methylation of *mir-34b/c* has also been reported in colorectal cancer (Toyota et al., 2008), oral squamous cell cancer (Kozaki et al., 2008), melanoma, and breast cancer (Lujambio et al., 2008). Thus, the epigenetic inactivation of *mi-34*s is a common event in human cancer.

mir-34a and *mir-34b/c* are intergenic miRNAs, and their expressions are regulated by the DNA methylation of their own promoters. Importantly, many miRNA encoding sequences are located within the introns of protein coding genes, and intronic miRNAs are usually expressed in a coordinate manner along with their host gene mRNAs (Baskerville and Bartel, 2005). We previously reported that *mir-126*, which is located within the intron of *EGFL7*, is silenced by the DNA methylation of its host gene in NSCLC (Watanabe et al., 2012). *mir-126* functions as a tumor-suppressive miRNA, suppressing metastasis in breast cancer (Tavazoie et al., 2008) and inhibiting the invasion of NSCLC cell lines by targeting *Crk* (Crawford et al., 2008). Moreover, *mir-126* was recently reported to be an essential regulator of angiogenesis. *Vascular endothelial growth factor (VEGF)-A* is a target of *mir-126*, and the downregulation of *mir-126* increases the activity of VEGF-A in lung cancer (Liu et al., 2009). A reduction in *mir-126* expression is significantly associated with increased microvessel density in oral squamous cell cancer (Sasahira et al., 2012) and NSCLC (Jusufovic et al., 2012), suggesting a negative regulatory role of *mir-126* in tumor angiogenesis. In addition, decreased *mir-126* expression is significantly associated with a shorter survival period in patients with NSCLC (Jusufovic et al., 2012). In contrast, Donnem et al. demonstrated that an elevated level of *mir-126* expression is associated with a shorter survival period in patients with NSCLC and that an elevated level of *mir-126* expression was associated with an increase in *VEGF-A* expression in

Table 1 | miRNAs silenced by DNA methylation in lung cancer.

miRNA	Target genes	Reference
mir-9-3		Lujambio et al. (2008), Kitano et al. (2011), and Heller et al. (2012)
mir-34a, -34b/c	Bcl-2, Cyclin D1, Cyclin E2, CDK4, CDK6, c-Myc, c-Met	Lodygin et al. (2008), Lujambio et al. (2008), Gallardo et al. (2009), Wang et al. (2011), Tanaka et al. (2012), and Watanabe et al. (2012)
mir-124-1, -124-2, -124-3	CDK6	Lujambio et al. (2007) and Kitano et al. (2011)
mir-126	Crk, VEGF-A	Watanabe et al. (2012)
mir-148a	TGIF2	Lujambio et al. (2008) and Chen et al. (2013)
mir-193a		Heller et al. (2012)
mir-200, -205	ZEB1, ZEB2	Tellez et al. (2011)
mir-487b	SUZ12, BMI1, WNT5A, MYC, K-ras	Xi et al. (2013)

NSCLC (Donnem et al., 2011). The targeted deletion of *mir-126* in mice impaired normal angiogenesis, suggesting a positive regulatory role of *mir-126* in angiogenesis (Wang et al., 2008). Additional research is required to elucidate the relation between *mir-126*, tumor angiogenesis, and tumor progression. Furthermore, we reported that the DNA methylation of *EGFL7* (a host gene of *mir-126*) was only observed in 7% of the clinical samples that were evaluated (Watanabe et al., 2012), which cannot completely explain the frequent downregulation of this miRNA in NSCLC. In fact, our analyzes of DNA methylation of the coding genes (Sano et al., 2007; Kusakabe et al., 2010) and miRNAs (Kitano et al., 2011; Watanabe et al., 2012) revealed that the ratio of DNA methylation is often quite low in primary NSCLCs. This low ratio of DNA methylation may be a result of insufficient DNA methylation following changes in histone modification, rather than the result of the coexistence of non-cancerous tissues, and may be responsible for the low frequency of DNA methylation of *mir-126* in primary NSCLCs if the method used to detect DNA methylation is not sufficiently sensitive.

Cigarette smoking is the most important risk factor for lung cancer. Accumulating evidence suggests that tobacco induces the epigenetic silencing of certain miRNAs in lung carcinogenesis (Tellez et al., 2011; Xi et al., 2013). Tellez et al. reported that the exposure of human bronchial epithelial cells (HBECs) to tobacco carcinogens decreased the expressions of *mir-200b*, *-200c*, and *-205* and induced the epithelial-to-mesenchymal transition (EMT). The *mir-200* family and *mir-205* function as key negative regulators of the EMT through the direct targeting of *ZEB1* and *ZEB2* (Gregory et al., 2008; Park et al., 2008). In HBECs, tobacco carcinogens initially induced an increase in H3K27me3 (inactive closed chromatin) and subsequently induced the DNA methylation of sequences encoding these miRNAs. The loss of *mir-200c* expression as a result of DNA methylation has been shown to induce an aggressive, invasive, and chemoresistant phenotype of NSCLC (Ceppi et al., 2010). In addition, Shien et al. (2013) demonstrated that a lung cancer cell line with acquired resistance to an EGFR tyrosine kinase inhibitor exhibited EMT features and the downregulation of *mir-200c* as a result of DNA methylation. Xi et al. reported that tobacco carcinogens induced the epigenetic silencing of *mir-487b* and that *mir-487b* functions as a tumor suppressive miRNA in NSCLC by targeting *SUZ12*, *BMI1*, *WNT5A*, *MYC*, and *K-ras*. These studies highlight the potential of epigenetic drugs to reverse tobacco-induced reprogramming in lung cancer cells.

H3K27me3 AND miRNAs

Epigenetic silencing in mammalian cells is mediated by at least two distinct histone modifications: histone H3 trimethylation at lysine 27 (H3K27me3) and histone H3 dimethylation and trimethylation at lysine 9 (H3K9me2 and H3K9me3). A recent genome-wide study of histone modifications in prostate cancer cells revealed H3K27me3 as a mechanism of tumor-suppressor gene silencing in cancer that occurs independently of promoter DNA methylation (Kondo et al., 2008). A polycomb group protein, EZH2, exhibits histone methyltransferase activity with substrate specificity for H3K27 (Cao and Zhang, 2004). EZH2 overexpression is associated with a poor prognosis in lung cancer, and the knockdown of EZH2 expression decreases the growth and invasion of lung cancer cells (Huqun et al., 2012). These findings suggest that aberrant H3K27me3 contributes to tumor-suppressor gene silencing in lung cancer, but genome-wide analyzes of H3K27me3 in lung cancer have not been reported.

Recently, Au et al. (2012) analyzed the changes in miRNA expression profiles induced by EZH2 knockdown and found that some tumor-suppressive miRNAs (*mir-139*, *-125b*, *-101*, *-200b*, and *let-7c*) are silenced by H3K27me3 in hepatocellular carcinoma. In lung cancer, *mir-212* is silenced by histone modifications rather than DNA methylation (Incoronato et al., 2011). Incoronato et al. showed that increases in H3K27me3 and H3K9me2 are observed in the *mir-212* promoter region in the lung cancer cell line Calu-1, which has a low *mir-212* expression level. *EZH2* may exert its oncogenic function, at least in part, by silencing tumor-suppressive miRNAs, and further investigation is required to verify the association between H3K27me3 and miRNA expression in lung cancer.

miRNAs THAT TARGET EPIGENETIC MACHINERY

While miRNA expression is regulated by DNA methylation and histone modifications, genes encoding the epigenetic machinery are also targeted by miRNAs. The *mir-29* family is the prototype of such miRNAs (Fabbri et al., 2007). The mir-29 family is comprised of three miRNAs (*mir-29a*, *mir-29b*, and *mir-29c*) that are derived from two transcripts (*mir-29b-1/29a* on chromosome 7 and *mir-29b-2/29c* on chromosome 1). The *mir-29* family is highly expressed in normal tissues and is downregulated in many types of human cancers including lung cancer (Yanaihara et al., 2006; Xu et al., 2009). *mir-29a* reportedly functions as an anti-metastatic and anti-proliferative miRNA in lung cancer (Muniyappa et al., 2009). *mir-29b* has also been reported to function as an anti-metastatic miRNA in lung cancer through the regulation of the *Src-ID1* pathway (Rothschild et al., 2012).

Recently, the *mir-29* family was shown to directly target *DNMT3A* and *DNMT3B*, two enzymes involved in *de novo* DNA methylation (Fabbri et al., 2007). The expression of *mir-29*s is inversely correlated with that of *DNMT3A* and *DNMT3B* in lung cancer tissue, and the enforced expression of *mir-29*s in lung cancer cell lines restores the normal pattern of DNA methylation, induces the re-expression of methylation-silenced tumor suppressor genes (such as *FHIT* and *WWOX*), and inhibits tumorigenicity both *in vitro* and *in vivo*. *mir-29b* also induces *PTEN* expression through the downregulation of DNA methyltransferases (DNMTs) and the subsequent hypomethylation of the *PTEN* promoter in a lung cancer xenograft model (Li et al., 2012). Samakoglu et al. (2012) also report that a combination therapy consisting of an *EGFR* antibody with cisplatin and gemcitabine induces *mir-29b* expression, the downregulation of *DNMT3b*, and the hypomethylation of tumor-suppressor genes in a lung cancer xenograft model. *mir-29b* has also been shown to down-regulate *DNMT1*, an enzyme involved in the maintenance of DNA methylation, indirectly by targeting *Sp1*, a transactivator of the *DNMT1* gene in leukemia (Garzon et al., 2009).

In addition to DNMTs, miRNAs can also target histone modification enzymes. *mir-449a/b* is downregulated and directly targets *HDAC1*, a histone deacetylase in lung cancer (Jeon et al.,

2012). *mir-101* reportedly targets *EZH2*, and the genomic loss of *mir-101* leads to the overexpression of *EZH2* in prostate cancer cells (Varambally et al., 2008). These results clearly show a strong interplay between miRNA and the epigenetic machinery, providing new insights into the molecular mechanism of aberrant DNA methylation and histone modifications in cancer.

RNA EDITING OF miRNAs

The most prevalent type of RNA editing in humans is the deamination of adenosine to inosine in double-stranded RNA (A-to-I editing). This process is catalyzed by two family members of adenosine deaminases acting on RNA (ADAR): ADAR1 and ADAR2. All known A-to-I edited sites have been attributed to *ADAR1* or *ADAR2* (Zinshteyn and Nishikura, 2009). Inosine is present in mRNA at tissue-specific levels that are correlated with ADAR expression. Analyzes of the amount of inosine in various mammalian tissues has revealed that inosine is most abundant in the brain, where one inosine molecule is present for every 17,000 ribonucleotides of mRNA; the second highest frequency of inosine has been observed in the lung, where one inosine molecule is present for every 33,000 ribonucleotides of mRNA (Paul and Bass, 1998).

Recently, certain pri-miRNAs have been reported to be subject to A-to-I editing. Kawahara et al. examined 209 pri-miRNAs and identified 47 pri-miRNAs as the targets of A-to-I editing in the human brain (Kawahara et al., 2008), suggesting that miRNA editing could have a considerable impact on miRNA-mediated gene silencing. Most A-to-I editing of pri-miRNAs results in altered miRNA processing by Drosha and Dicer (Kawahara et al., 2008). However, in rare cases, such as *mir-376*, RNA editing causes base substitution in the seed sequence and generates edited mature miRNAs with unique target genes and functions different from those of the unedited miRNAs (Kawahara et al., 2007).

Emerging lines of evidence suggest a link between A-to-I editing and cancer. Anomalous ADAR activity in cancer may lead to alterations in the efficiency of A-to-I editing. For example, the *glutamate receptor subunit B* (*GluR-B*) is nearly 100% edited at one position (Q/R site) in the normal brain. In primary glioblastoma, this position is substantially under-edited, compared with normal tissues, because of the decreased activity of ADAR2, which is responsible for the A-to-I editing of *GluR-B* (Maas et al., 2001). Recently, the efficiency of A-to-I editing of *mir-376a** was found to be significantly attenuated in glioblastoma cells (Choudhury et al., 2012). Unedited mir-376a* promotes the migration and invasion of glioma cells, whereas edited mir-376a* suppresses these features. These results suggest that the attenuation of A-to-I editing of *mir-376a** promotes the invasiveness of glioblastoma. Considering the relatively high prevalence of inosine in lung mRNA and the frequent A-to-I editing of miRNAs in the brain, it would be tempting to conduct a large-scale survey to evaluate the A-to-I editing of pri-miRNAs in normal lung and to analyze the alteration of miRNA editing and ADAR activity in lung cancer.

CONCLUSIONS AND FUTURE PERSPECTIVES

The two major challenges in studying the role of miRNA in cancer are (i) the identification of target genes, and (ii) the elucidation of the mechanisms that regulate miRNA expression in both normal and cancer cells. Limited knowledge is available regarding miRNA transcription, primarily because of inadequate information on the precise locations of the promoters and transcriptional start sites of the miRNAs. Approximately half of all miRNAs are intragenic sequences that are located within the exons, introns, or 3′UTRs of protein-coding genes. These intragenic miRNAs share promoters with their host genes and are co-regulated with their host genes, as in the case of mir-126. The remaining 50% of miRNAs are intergenic miRNAs with their own promoters, which have not been experimentally validated in most cases. Comprehensive analyzes of the miRNA transcription unit will help to elucidate the transcription factors or epigenetic changes responsible for alterations in miRNA expression in cancer.

The impairment of the miRNA biogenesis pathway and the attenuation of A-to-I editing add to the growing complexity of miRNA deregulation in cancer. Moreover, 3′UTRs of certain mRNAs are progressively shortened in cancer cells as a result of changes in alternative cleavage and polyadenylation (APA), a phenomenon that alters the 3′UTR length. Progressive 3′UTR shortening in cancer cells may lead to the disruption of miRNA-mediated gene silencing (Mayr and Bartel, 2009). Understanding these complexities as well as those of miRNA transcriptional regulation may lead to the identification of novel biomarkers and should help to unravel the impact of miRNA in lung carcinogenesis.

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The role of mesenchymal stem cells in cancer development

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The role of mesenchymal stem cells (MSCs) in cancer development is still controversial. MSCs may promote tumor progression through immune modulation, but other tumor suppressive effects of MSCs have also been described. The discrepancy between these results may arise from issues related to different tissue sources, individual donor variability, and injection timing of MSCs. The expression of critical receptors such as Toll-like receptor is variable at each time point of treatment, which may also determine the effects of MSCs on tumor progression. However, factors released from malignant cells, as well as surrounding tissues and the vasculature, are still regarded as a "black box." Thus, it is still difficult to clarify the specific role of MSCs in cancer development. Whether MSCs support or suppress tumor progression is currently unclear, but it is clear that systemically administered MSCs can be recruited and migrate toward tumors. These findings are important because they can be used as a basis for initiating studies to explore the incorporation of engineered MSCs as novel anti-tumor carriers, for the development of tumor-targeted therapies.

Keywords: stem cell transplantation, epithelial mesenchymal transition, cancer therapy, cytokines, stem cell niche

INTRODUCTION

Mesenchymal stem cells (MSCs) belong to a category of clinically relevant cell types that have the potential to be utilized for cell-based therapies, because complicated culturing or handling techniques are not required to yield clinically practical quantities. Traditionally, MSCs can be induced to differentiate into mesenchymal lineages such as osteoblasts, adipocytes, chondrocytes and potentially other skeletal tissue cells by culturing MSCs under defined mechanochemical conditions (Pittenger et al., 1999). MSCs are characterized by the expression of cell surface markers such as CD73, CD90, and CD105, and the absence of expression of hematopoietic lineage markers (Lama et al., 2007). Recently, there has been heightened interest into the homing and migration capacity of MSCs into tumors. Since the process of tumor progression is closely related to inflammation, the role of MSCs in carcinogenesis has emerged as an attractive new concept in cancer therapy. Although ample experimental evidence exists in support of the therapeutic potential of MSCs targeting different tumors, e.g., hepatocellular carcinoma (Gao et al., 2010), brain tumors (Nakamizo et al., 2005) and sarcoma (Khakoo et al., 2006) in different animal models, the mechanisms guiding the homing and recruitment of MSCs into tumors and their potential role in malignant tissue progression are not well understood. MSCs have been shown to promote tumor progression through immune modulation (Karnoub et al., 2007). In contrast, other studies have reported that MSCs have a suppressive effect on tumor development; e.g., via modification of Akt signaling (Khakoo et al., 2006). The use of different tissue sources, individual donor variability, and injection timing of MSCs in each experiment may have an impact on this discrepancy. The expression of critical receptors such as Toll-like receptor (TLR) is variable at various time points during the treatment (Liotta et al., 2008), which may also influence the effects of MSCs on tumor progression. Whether MSCs support or suppress tumor progression, it is clear that systemically administered MSCs can be recruited by, and migrate toward, tumors (Studeny et al., 2002; Loebinger et al., 2009). These findings are important because they can be used as a basis for studies to explore the utilization of engineered MSCs as novel carriers for delivery of anti-tumor agents to cancerous tissue, guiding the development of tumor targeted therapies.

Previous reports (Curtin et al., 2009; Tang et al., 2010) have led to a great deal of attention into the role and function of MSCs in tumors. The tropism of MSCs for tumors raised wide interest regarding their potential as a delivery vehicle for anti-cancer agents. Indeed, several reports have described the feasibility of using these cells as anti-cancer delivery vehicles because they secrete various anti-cancer molecules such as tissue necrosis factor (TNF), TNF related apoptosis inducing ligand (TRAIL), and interferon (IFN)-β by transfection. These studies demonstrated a sufficient effect in suppressing tumor progression (Nakamizo et al., 2005; Loebinger et al., 2009; Grisendi et al., 2010). However, Karnoub et al. (2007) suggested that MSCs have a supportive effect on tumor progression showing that co-injection of MSCs with cells from a breast cancer cell-line led to a higher degree of metastasis, but this effect was not significant in local tumor growth. This supportive effect on tumor growth by MSCs has also been reported in different cancers such as colon cancer, lymphoma, and melanomas (Djouad et al., 2003; Ame-Thomas et al., 2007; Shinagawa et al., 2010). In contrast, several reports showed that MSCs may have a suppressive role in tumor development via p38 mitogen-activated protein kinase (MAPK; Tian et al., 2010) or by cell fusion (Wang et al., 2012). Also, different types of tumors such as liver cancer, breast cancer, leukemia, and pancreatic cancer have been used to show a tumor suppressive effect of MSCs (Qiao et al., 2008a,b; Cousin et al., 2009; Zhu et al., 2009). On the other hand, Torsvik et al. (2010) suggested that cross-contamination of MSCs

with tumor cells can enhance tumor supportive behavior. Interestingly, Klopp et al. (2011) suggested that the increased tumor mass observed in these reports can be related to increased proliferation of MSCs in the tumor.

In this review, we discuss recent findings related to mechanisms of MSC migration toward tumors, including cytokines, chemokines, as well as surrounding conditions, and, more importantly, we will also discuss the potential role of MSCs in malignant tissue progression.

THE MESENCHYMAL STEM CELL NICHE *IN VIVO*

It was previously revealed that the primary MSC niche is in bone marrow; however, there are several reports that also identify additional peripheral locations, such as adipose tissue, salivary glands, tendon, periodontal ligament, skin, muscle, lung and, most recently, Powell et al. (2011) reported that the intestinal lamina propria is also a niche. A report by da Silva Meirelles et al. (2008) posits that an important MSC niche is the perivascular region, which, as a residual aspect of embryogenesis, might explain the presence of MSCs in many different tissue types. Stappenbeck and Miyoshi (2009) have suggested that MSCs might originate in the bone-marrow and, subsequently, be recruited distally to specific sites of tissue injury). Nonetheless, there appear to be two origins of MSC populations. One population present in peripheral locations where they interact with perivascular cells (an embryonic remnant), and a second population originating in the bone marrow, where MSCs form their primary stem cell niche and respond appropriately following tissue injury. MSCs secrete various families of active molecules, including cytokines, chemokines, and growth factors, which regulate the local bone marrow environment and modulate systemic immune responses to inflammatory events. Although there have been numerous reports demonstrating that MSCs can repair tissue by directly differentiating toward mesenchymal lineages, recent work has established that instead of, or perhaps in addition to differentiation, MSCs can enhance the differentiation of other progenitor cells into functional somatic cells. In addition, they may contribute to other aspects of local tissue repair via paracrine mechanisms.

Mesenchymal stem cells can function as immune suppressive and anti-inflammatory agents, as well as stimulators of tissue repair and regeneration. However, the difference between MSCs that are recruited from the bone marrow versus peripherally located MSCs in executing these distinct roles is unclear. Recently, Brandau et al. (2010) compared the differentiation potentials of local resident and bone marrow-derived MSCs, and suggested that the two populations were not identical. Indeed, da Silva Meirelles et al. (2008) demonstrated differences in the degree of differentiation among MSCs originating from different tissues. The homing capacity toward cancer tissues has been evaluated mostly in MSCs derived from bone marrow (Hung et al., 2005; Nakamizo et al., 2005; Loebinger et al., 2009) and less from other tissues such as adipocytes (Grisendi et al., 2010) or umbilical cord blood (Hu et al., 2011). However, once incorporated into a tumor, MSCs might contribute with other cells such as myofibroblasts, endothelial cells, pericytes, and inflammatory cells to create a microenvironment that mirror the environment of a chronic wound (Dvorak, 1986; Bergfeld and DeClerck, 2010). In this context, local tissue derived MSCs, such as from pericytes, might contribute to tumor progression.

THE IMPACT OF ROLE DISCREPANCY ON MESENCHYMAL STEM CELLS IN CANCER DEVELOPMENT

Resident MSCs may have a critical role in maintaining the homeostasis of injured tissue through immune modulatory effects or angiogenic stimulation by secreting bioactive molecules (Lazennec and Jorgensen, 2008; Uccelli et al., 2008). Since the actual population of resident MSCs is thought to be very low and decreases with age (Caplan, 2007), the behavior of large quantities of experimentally administered MSCs is likely to be quantitatively different from the behavior of the small amount of resident MSCs. Therefore, the role of administered MSCs in cancer development is still controversial. There are various reports that describe the ability of MSCs to promote tumor progression by enhancing metastatic potential (Karnoub et al., 2007) as well as epithelial mesenchymal transition (EMT; Kabashima-Niibe et al., 2013). In contrast, Ho et al. (2013) reported tumor suppressive effects of MSCs via modification of Akt signaling, shown by the coadministration of MSCs and glioma cells resulting in a significant reduction in tumor volume and vascular density. Also, several reports have demonstrated a suppressive effect of MSCs on different types of tumors (Otsu et al., 2009; Dasari et al., 2010). These conflicting results may be due to variable experimental factors such as differences in cell source like bone marrow or fat tissue, different time points, method of cell administration, and timing. In addition, although the composition of culture media is similar to fluids present *in vivo*, it does not supply all of the bioactive factors present in the stem cell niche (Watt and Hogan, 2000). Therefore, cultured MSCs should not be considered equivalent to MSCs under physiological conditions *in vivo*. Interestingly, resident MSCs, derived from either bone marrow or local tissues, have been reported to partly contribute to the origin of cancer-associated fibroblast (CAFs) or tumor-associated myofibroblasts (Quante et al., 2011). Using a mouse model of infiammation-induced gastric cancer, Quante et al. (2011) showed that at least 20% of CAFs originate in bone marrow and are derived from MSCs. This study suggested that the number of MSCs could increase in response to cancer development and promote the malignant potential.

Whether MSCs support or suppress tumor progression, it is clear that systemically administered MSCs can be recruited and migrate toward tumors (Koc et al., 2000; Nakamizo et al., 2005). Although the effect on the tumor might be enhanced by timing or the number of administered cells, these findings can be used for the development of tumor targeted therapies by providing a basis for conducting studies to explore the incorporation of engineered MSCs as novel anti-tumor carriers (Reagan and Kaplan, 2011).

HOMING MECHANISM OF MSCs TOWARD CANCER

Recently, interest into our understanding of MSC homing and migration into tumors has grown, and several investigators have begun to compare these two processes. Since the process of tumor progression is highly related to inflammation and, as reported by Kalluri and Weinberg (2009) that EMT is critical in cancer development, the role of MSCs in carcinogenesis is an attractive

new concept in cancer therapy. In this section, we review recent reports demonstrating the homing mechanisms of MSCs. Several different mediators have been reported to be involved in this process. Some of these molecules are growth factors, chemokines and cytokines, which can regulate cell migration toward inflammatory sites. These include SDF-1 and SCF-1, CCL5/CCR5, CCR2, TNF-α, and other peptides.

GROWTH FACTORS

Vascular endothelial growth factor (VEGF) seems to be one of the most important factors that enhances and directs stem cell motility. Indeed MSCs demonstrate intensive migratory and invasive behavior in the presence of gliomas, which express high levels of VEGF. It was reported by Ritter et al. (2008) that VEGF, as well as bFGF secreted by breast cancer cells, induced the migration of MSCs. They also demonstrated that receptors for these molecules were expressed on MSCs and that depletion of these growth factors using antibodies reduced MSC migration capacity. In addition, MSCs have been shown to migrate toward endothelial cell-derived capillaries and inhibit tumor angiogenesis (Otsu et al., 2009). Although VEGF might contribute to this phenomenon, MSCs have been shown to have opposing effects on tumors. MSCs are known to express EGF and PDGF receptors on their surface, and antibodies that block PDGF or EGF can attenuate the migration of MSCs. Those reports demonstrated that certain malignant tumors such as in glioma and breast cancer, which have highly specialized vasculature and stroma, can provide permissive environments for the selective engraftment of MSCs. Taken together, the tropism of MSCs for tumors may be mediated, at least in part, by specific growth factors and receptors expressed by MSCs, thereby using a recruitment mechanism similar to what is used in inflamed or injured tissue.

CHEMOKINES AND CYTOKINES

Stromal cell derived factor 1α (SDF-1α), which is a well-established chemo-attractant for leukocytes, acts directly on cancer cells by stimulating proliferation through the SDF-1α receptor chemokine (C-X-C motif) receptor 4 (CXCR4) expressed on cancer cells. However, SDF-1α secretion also leads to recruitment of endothelial progenitor cells to the growing tumor, thereby promoting angiogenesis. Some reports have demonstrated that SDF-1α secreted from cancer cells enhanced MSC tropism, and an antibody that blocks SDF-1α attenuated the migration of MSCs. Infection-related cancer development processes, such as helicobacter infection in gastric cancer, can give rise to an environment conducive to the recruitment of MSCs, and this can be regulated by SDF-1 and SCF-1. It was also reported that in response to chronic helicobacter infection, bone marrow–derived cells could home to and repopulate the gastric mucosa and contribute over time to metaplasia, dysplasia, and cancer (Stoicov et al., 2013).

Chemokine (C-C motif) ligand 5 (CCL5) is one of the chemokines that can enhance stem cell migration during inflammation. It was reported by Karnoub et al. (2007) that actions of CCL5 were responsible for MSC-induced metastasis in breast cancer cells. Breast cancer cells stimulated *de novo* secretion of the chemokine CCL5 (also called RANTES) from MSC, which then acted in a paracrine fashion on cancer cells to enhance their motility, invasion and metastasis (Karnoub et al., 2007). This result shows the critical importance of CCL5–CCR5 paracrine interactions in enabling MSCs to induce cancer metastasis.

MSC RESPONSE TO IRRADIATION AND HYPOXIA

It was reported by Klopp et al. (2007) that the role of inflammation-related cytokines and chemokines in radiation-enhanced MSC migration is significant. They identified cytokines and chemokines involved in chemotaxis towards irradiated tumor microenvironments. Low-dose irradiation of murine tumors enhanced the tropism for, and engraftment of, MSCs in irradiated tumor environments. They demonstrated that tumor cells were able to increase the production and secretion of cytokines following irradiation, e.g., VEGF, PDGF, and TGF-β, that enhanced the migratory properties of MSCs (Klopp et al., 2007). In addition, the chemokine receptor CCR2 was found to be up-regulated in MSCs exposed to irradiated tumor cells. CCR2 was undetectable or expressed at low levels in untreated cells but could be up-regulated by inflammatory cytokines such as TNF-α. In addition, inhibition of CCR2 led to a marked decrease of MSC migration *in vitro* (Ren et al., 2012). Taken together, these experiments suggest that radiation can increase the expression of inflammatory mediators that can secondarily enhance the recruitment of MSCs into the tumor microenvironment.

Hypoxia also plays a major role in tumor progression, metastasis, and poor clinical outcomes. However, the role of hypoxia in MSC recruitment to the tumor microenvironment has not been sufficiently described. It was reported by Rattigan et al. (2010) that hypoxic breast cancer cells enhance their production of IL-6, which promotes the recruitment of MSCs. Secreted IL-6 acts in a paracrine fashion on MSCs, stimulating the activation of both Stat3 and MAPK signaling pathways to enhance migratory potential and cell survival. Specifically, increased cellular migration is dependent on IL-6 signaling through the IL-6 receptor (Rattigan et al., 2010). IL-6 is generally thought of as a multifunctional cytokine that plays a role in apoptosis, cell proliferation and survival. It binds to its cognate receptor, leading to activation of the JAK/STAT signal transduction pathway. A similar pathway may contribute to the migratory capacity of MSCs toward hypoxic tumors.

OTHER EXOGENOUS MOLECULES

Previous studies have shown that leucine, leucine-37 (LL-37), the C-terminal peptide of human cationic antimicrobial protein 18, stimulates the migration of various cell types and has similar expression patterns in tumors, damaged tissue, and in inflammatory tissue, where MSCs are prominent. These peptides also have the ability to stimulate chemotaxis of various cell types. It was reported by Coffelt et al. (2009) that LL-37 promotes ovarian tumor progression through recruitment and engraftment of MSCs into tumors, where these cells provide pro-angiogenic and immunomodulatory factors to support tumor growth and progression. Indeed, neutralization of LL-37 *in vivo* significantly reduces the engraftment of MSCs into ovarian tumor xenografts, resulting in inhibition of tumor growth, as well as disruption of the fibrovascular network. LL-37-mediated migration of MSCs

into tumors likely occurs through formyl peptide receptor like-1 (Coffelt et al., 2009). These data indicate that LL-37 facilitates tumor progression through recruitment of progenitor cell populations to serve as pro-angiogenic factor-expressing tumor stromal cells. Expressed factors include IL-1 receptor antagonist, IL-6, IL-10, CCL5, VEGF, and matrix metalloproteinase-2 (MMP-2). The overall consequence of LL-37's actions, through its recruitment of MSCs, is advancement of tumor progression.

Urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor (uPAR) are up-regulated in tumors of various origins, where they play critical roles in the development of invasive and chemo-resistant cancer phenotypes. The activation of uPA and uPAR in malignant solid tumors (brain, lung, prostate, and breast) augments MSC tropism. It was reported by Gutova et al. (2008) that chemo-attraction of MSCs to cancer cells is strongly correlated with uPAR expression levels in tumor cells, which may be important for the development of optimal stem cell-based therapies.

DEVELOPMENT OF MSCs AS A STEM CELL THERAPY

Since it was reported by Tolar et al. (2007) that sarcoma developed following transplantation of MSCs into animals, determination of their therapeutic efficacy and safety is now required for clinical applications. From a practical perspective, MSCs seem to be a very promising cell source for use in stem cell therapies for tissue impairment, given that MSCs can home to inflamed or injured tissues, as well as tumors, likely without differentiating into somatic cells. It is important to identify the utility of MSCs in clinical settings, in the context of an understanding of their complicated mechanisms as immune and inflammatory regulators. As discussed in this chapter, the most promising clinical aspects of MSCs might be immune-modulatory and anti-inflammatory effects. However, major challenges remain in our understanding of both the actual benefits, as well as the side effects of these cells in human disease.

This review discussed key modulators regarding the importance of the migration capacity of MSCs. Controlling the level of these key factors in target tissues may be a way to increase the specificity of MSC applications in these tissues, which may also lead to a reduction in the total cell number needed for the therapy, and, in concert, may reduce potential side effects, such as malignant transformation. Receptors for the reviewed key factors expressed on MSCs, including TLR and CXCR4, can also be potentially modified genetically via transfection, which may augment the efficacy of MSCs in clinical settings and decrease the migration of MSCs to non-targeted sites.

However, the clinical application of MSCs for cancer treatment is still challenging. This review described the migratory potential of MSCs to malignant tissues, which is largely similar to MSC migration into inflammatory tissue. However, factors released from malignant cells, as well as surrounding tissues and the vasculature, are still regarded as a "black box." Thus, remains difficult to provide a specific role for MSCs in cancer development after they migrate and home into different tissues. Although some reports have demonstrated a tumor suppressive effect of MSCs, others described a tumor supportive potential. In any case, these reports encourage the notion that MSCs may play a critical role in cancer development and may be useful as a novel therapeutic delivery system that can target malignant tumors, potentially superior to existing therapeutic molecular therapies. While MSCs can react to surrounding microenvironments, molecular therapies cannot. Thus, it is imperative that scientists continue to investigate the roles and mechanisms of MSCs in tumor progression in order to harness the therapeutic potential of MSCs to regulate both inflammatory and metastatic diseases.

For clinical applications, the methodology of administration of MSCs is crucial to determine their efficacy, since there are several reports describing the risk of capillary embolism by MSCs after intravascular administration (Furlani et al., 2009; Tatsumi et al., 2013). Additional strategies, such as co-administration of anti-coagulant or adhesion factors (Tatsumi et al., 2013), as well as engineering approaches (Karoubi et al., 2009; Houtgraaf et al., 2013), might attenuate these risks.

CONCLUSION

In summary, there are a variety of studies that demonstrate the potential migration of MSCs toward and into tumors in response to multiple molecules secreted from tumors as well as surrounding tissues. These findings will help to clarify the role of MSCs in tumors, as well as the key mechanisms that determine whether MSCs are suppressive or supportive for tumor growth. Since MSCs are relatively easy to obtain and grow in the laboratory, we anticipate that this review may help to develop new approaches in cell therapy using MSCs to control cancer development.

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