Review

Secretory MicroRNAs by Exosomes as a Versatile Communication Tool

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Abstract: In the past several years, the importance of microRNA (miRNA) in cancer cells has been recognized. Proper control of miRNA expression is essential for maintaining a steady state of the cellular machinery. Dysregulation of miRNAs leads to the cancer development, meaning that expression profile of miRNAs can be used as cancer biomarker, and recovery of down-regulated miRNAs or inhibition of up-regulated miRNAs will be a novel approach for cancer therapy. Recently, it was discovered that extracellular miRNAs circulate in the blood of both healthy and diseased patients. Most of the circulating miRNAs are included in protein, lipid or lipoprotein complexes, such as RNA-binding proteins, apoptotic bodies, microvesicles, or exosomes, and are, therefore, highly stable. The existence of circulating miRNAs in the blood of cancer patients has raised the possibility that miRNAs may serve as a novel diagnostic marker. However, the secretory mechanism and biological function, as well as the meaning of the existence of extracellular miRNAs, remain largely unclear. Our recent study revealed the secretory mechanism of miRNAs and showed their cell-to-cell transfer. Here we summarize current approaches to modulate the intercellular and interindividual network via silencing signals exported by secretory miRNAs and discuss about the usage of circulating miRNAs as a novel communication tool.

Key words: microRNA, exosome, diagnosis, micro-environment, communication.

Finding of secreting microRNAs in body fluids

Growing evidence suggests that extracellular microRNAs (miRNAs) stably exist in human body fluids, including plasma, saliva, and urine, although ribonucleases (RNases) also circulate throughout the body.[1] This finding indicates that miRNAs are excreted after they are contained in RNase-resistant lipid vesicles, such as exosomes and apoptotic bodies. Recent studies have revealed the novel genetic exchange between cells using miRNA either in microvesicles (up to 1 μm) or in small membrane vesicles of endocytic origin called exosomes (50~100 nm).[2~11] One of the first reports showing the existence of miRNA in exosomes was studied by Valadi et al., who reported that exosomes released from human and murine mast cell lines contain mRNAs and miRNAs.[11] Hunter et al. demonstrated that miRNAs contained in the microvesicles from blood were known to regulate the cellular differentiation of blood cells and metabolic pathways and to modulate immune function.[12] Apart from microvesicles and exosomes, more recent evidence has suggested that circulating microRNAs found in sera of the human are coupled with some specific proteins and lipoproteins. However, very little is known about the secretory machinery of miRNAs.

Oral cancer miRNAs in saliva

Considering that exosomes and microvesicles are evident in several types of body fluid from cancer patients, miRNA surely be able to be found not only in serum/plasma but also in other body fluid. Indeed, Michael et al. showed the presence of miRNAs within exosomes isolated from human saliva.[13] Furthermore, analyzing patient saliva with a polymerase chain reaction (PCR) technique, Park et al. found that miR-125a and miR-200a were present in significantly lower levels

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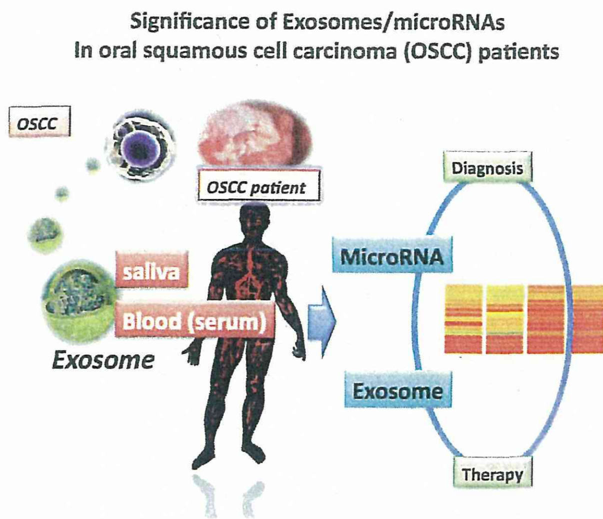

Fig. 1 Significance of Exosomes/microRNAs in oral squamous cell carcinoma (OSCC) patients. Circulating microRNAs in OSCC patients-exosomes (saliva and serum) are useful for a novel diagnosis and therapeutics against OSCC.

in the saliva of oral squamous cell carcinoma (OSCC) patients than in control subjects.[14] These findings suggest that the detection of miRNAs in saliva can be used as a noninvasive and rapid diagnostic tool for the diagnosis of oral cancer (Fig. 1). OSCC is the sixth most common cancer in the U.S., accounting for 90% of oral cancers and leading to 8,000 deaths per year. The average five-year survival rate for OSCC is about 50%, and this number has not changed in last three decades. Therefore, an early detection method for OSCC is needed to increase long-term patient survival. In this connection, finding of OSCC specific miRNAs highlighted a novel means of early cancer detection, and that the presence of salivary miRNA adds a third type of molecule, in addition to proteome and transcriptome, that can be measured in human saliva.

Exosomes in saliva

As human plasma, saliva contain exosomal particles. Saliva from healthy human donors was collected in tubes. For the RNA isolation, 100 μl of the protease inhibitor and RNase inhibitor were added per 20 ml of saliva. The saliva was diluted 1:1 with phosphate buffered saline (PBS) and centrifuged at 20,000×g for 20 min to remove cells and cell debris. The supernatant was filtered through a 0.2 μm filtration system and then ultracentrifugation at 100,000×g for 70 min to pellet the exosomes. By electron microscopic analysis, salivary exosomes look like round shape with 120~150 nm in a diameter. It is reported that exosomes from saliva can be taken up by human macrophages, as shown by the uptake of fluorescently stained exosomes.[15] It has been shown that other cells can take up exosomes in a similar way to macrophages, which means that this is a common feature of exosomes. It has not been determined the cellular origin of saliva exosomes, but it has been shown that primary cultures of salivary glands can release exosomes-like particles suggesting that exosomes in saliva are partly derived from salivary gland epithelial cells.

Molecular mechanisms of secreting miRNAs and exosomes

Our group demonstrated that the secretion of miRNAs depends on the cellular amount of ceramide, a bioactive sphingolipid, whose synthesis is tightly regulated by neutral sphingomyelinase 2 (nSMase2).[16] Treatment of a chemical compound, GW4869, which can inhibit the enzymatic activity of nSMase2, markedly blocked the secretion of miRNAs and exosomes. These data suggest that miRNAs are secreted by an exosome-dependent pathway that involves ceramide biosynthesis. The molecular dissection of miRNA secretory mechanisms is likely to assist in a better understanding why the expression of secretory miRNAs is perturbed during the development of various diseases, including cancer, diabetes, and immune disorders.[17~19] This finding may be beneficial to confer reliability and credibility to the diagnostic use of secretory miRNAs.

Intracellular communication by exosomes

As evidenced by many reports,[20~24] small RNAs are currently regarded as a category of intercellular signal entities, which are called, for instance, mobile small RNAs, systemic silencing signals, or just secreted RNAs. To answer the question as to whether

the secretory exosomal miRNAs that we observed can function biologically in a similar manner to other members, we set up *in vitro* and *in vivo* experiments pertaining to intercellular transfer. Purified exosomes labeled with a green fluorescent reagent PKH67 were successfully incorporated into recipient PC-3 cells. We also detected the migration of secretory nucleic acids into PC-3 cells by using SYTO dye, a specific probe for vital DNAs and RNAs including mRNAs and miRNAs. Furthermore, we reported that secretory miR-146 inducing a phenotypic change in the incorporated cells. It is generally acknowlkedged that normal epithelial cells regulate the secretion of autocrine and paracrine factors that prevent aberrant growth of neighboring cells, leading to healthy development and normal metabolism. One reason for tumor initiation is considered to be a failure of this homeostatic cell competitive system. Kosaka et al. identify tumor-suppressive miRNAs secreted by normal cells as anti-proliferative signal entities.[25] Among these miRNAs, secretory miR-143 could induce growth inhibition exclusively in cancer cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process. Taken together, the findings indicate that exosomal secretory miRNAs can spread translation-inhibitory signals, leading to the elicitation of a wide array of biological events.

Secretory miRNAs could be an interindividual communication tool

The relevance of miRNA transfer cannot be limited within an individual organism. Kosaka et al. found that the expression of immune-related miRNAs in human breast milk culminates in the first 6 months of lactation, in agreement with the significance of colostrum in the context of passive immunity.[26] This paper appears to suggest the two important concepts such as dietary intake of miRNAs and vertical transfer of miRNAs. If the miRNAs intake by daily food maintain their biological activity after digestion, they will be highly valued as a crucial ingredient that can modulate gene expression of our own cells. In fact, current reports suggested that plant (rice origin) miRNAs could found in the circulating blood.[27] The microRNAs seem to come from ingested rice. Presumably the micoRNAs are taken up in the intestine and secreted into the blood in small vesicles, since microRNAs packaged into exosomes are highly stable in low pH such as stomach acid. Based on the second concept, genetic exchanges between mother and child would be mediated by milk-exosomes as well as amniotic fluids. The excretions from the body, such as tears, saliva, semen, and vaginal discharge, also include secretory miRNAs, suggesting that these fluids may act as a mediator of horizontal genetic materials transfer. It is within the bounds of probability that secretory miRNAs could be an interindividual communication tool among humans.

Perspective

The existence of circulating miRNAs and micro-vesicles in the blood of cancer patients has raised the possibility that disease-specific miRNAs and exosomes may serve as a novel diagnostic marker. Moreover, exosomes secreted from tumor cells contribute maicro-managing tumor microenvironment. Intracellular communications via microRNAs and microvesicles including exosomes are important novel therapeutic targets for inhibiting tumor development and metastasis including oral cancer.

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Article

Genome-Wide Analysis of DNA Methylation and Expression of MicroRNAs in Breast Cancer Cells

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Abstract: DNA methylation of promoters is linked to transcriptional silencing of protein-coding genes, and its alteration plays important roles in cancer formation. For example, hypermethylation of tumor suppressor genes has been seen in some cancers. Alteration of methylation in the promoters of microRNAs (miRNAs) has also been linked to transcriptional changes in cancers; however, no systematic studies of methylation and

transcription of miRNAs have been reported. In the present study, to clarify the relation between DNA methylation and transcription of miRNAs, next-generation sequencing and microarrays were used to analyze the methylation and expression of miRNAs, protein-coding genes, other non-coding RNAs (ncRNAs), and pseudogenes in the human breast cancer cell lines MCF7 and the adriamycin (ADR) resistant cell line MCF7/ADR. DNA methylation in the proximal promoter of miRNAs is tightly linked to transcriptional silencing, as it is with protein-coding genes. In protein-coding genes, highly expressed genes have CpG-rich proximal promoters whereas weakly expressed genes do not. This is only rarely observed in other gene categories, including miRNAs. The present study highlights the epigenetic similarities and differences between miRNA and protein-coding genes.

Keywords: DNA methylation; microRNA; cancer

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1. Introduction

DNA methylation plays important roles in development, differentiation, X inactivation, genomic imprinting, and silencing of transposable elements through the regulation of transcription [1–3]. This usually occurs in mammalian cells at cytosine residues in the context of cytosine-phosphate-guanine dinucleotide (CpG), and approximately 60%–90% of cytosines at these sites are methylated [4]. Methyl-CpG serves as the physiological ligand for a family of proteins containing a highly conserved methyl-CpG binding domain (MBD) [5]. These proteins recruit various chromatin-modifying complexes to methyl-CpG sites to cause further chromatin structural changes that result in transcriptional silencing.

Alterations of the patterns of normal DNA methylation result in many human diseases, including cancer [6]. Aberrant patterns of DNA methylation in cancers are associated with tumor type, stage, prognosis, and response to chemotherapy. Human tumors undergo global DNA demethylation, including of Line-1 repetitive elements, and then DNA hypermethylation of certain gene promoters, including those of tumor suppressor genes [7]. Aberrant DNA methylation in cancers is not restricted to protein-coding genes; it is also observed in microRNA (miRNA) genes. MicroRNAs are small noncoding regulatory RNAs of 20–24 nucleotides that reduce the stability and/or translation of fully or partially sequence-complementary target mRNAs. MicroRNAs can act as oncogenes or tumor suppressors, and can contribute to cancer initiation and progression [8].

Genome-wide analysis of DNA methylation is an important issue in epigenetic research. The oldest technology for genome-wide analysis of DNA methylation, Restriction Landmark Genomic Scanning, which utilizes two-dimensional gel electrophoresis of genomic DNA, was developed in 1991 [9,10]. This technology has been used in the cloning of many imprinted genes [10] and tumor suppressors [11,12]. In this century, development of genome-wide analysis technology such as microarrays and next-generation sequencers brought about several new methods for analyzing DNA methylation [13]. Among these methods, capture and next-generation sequencing of methyl-CpG

binding domains of MBD1 protein is especially useful for analysis because the binding activity of this protein is stronger than that of antibodies [14].

Although epigenetic silencing of miRNAs has been reported in many cancers, systematic studies of DNA methylation and miRNA transcription have not yet been reported. In addition, differentially methylated regions were found only in the CpG islands just upstream of miRNAs in most reports on the epigenetic silencing of miRNAs because such cases are easily discovered. Therefore, it has not been clarified whether the transcriptional start sites far upstream of miRNAs are influenced by DNA methylation or not. It has also not been clarified whether the CpG-poor promoters of miRNA are influenced by DNA methylation. Furthermore, miRNAs are not only transcribed by RNA polymerase II, which is responsible for most protein-coding genes, but also transcribed by RNA polymerase III. Although the relation between DNA methylation and silencing has been extensively studied in genes transcribed by RNA polymerase II, there are few reports for genes transcribed by RNA polymerase III. Therefore, systematic analyses of the methylation and expression of miRNAs are required. To clarify the relation between DNA methylation and transcription of miRNAs, here we systematically analyzed the methylation and expression of human genes encoding miRNAs, proteins, other non-coding RNAs (ncRNAs), and pseudogenes using next-generation sequencing and microarray analysis.

2. Results and Discussion

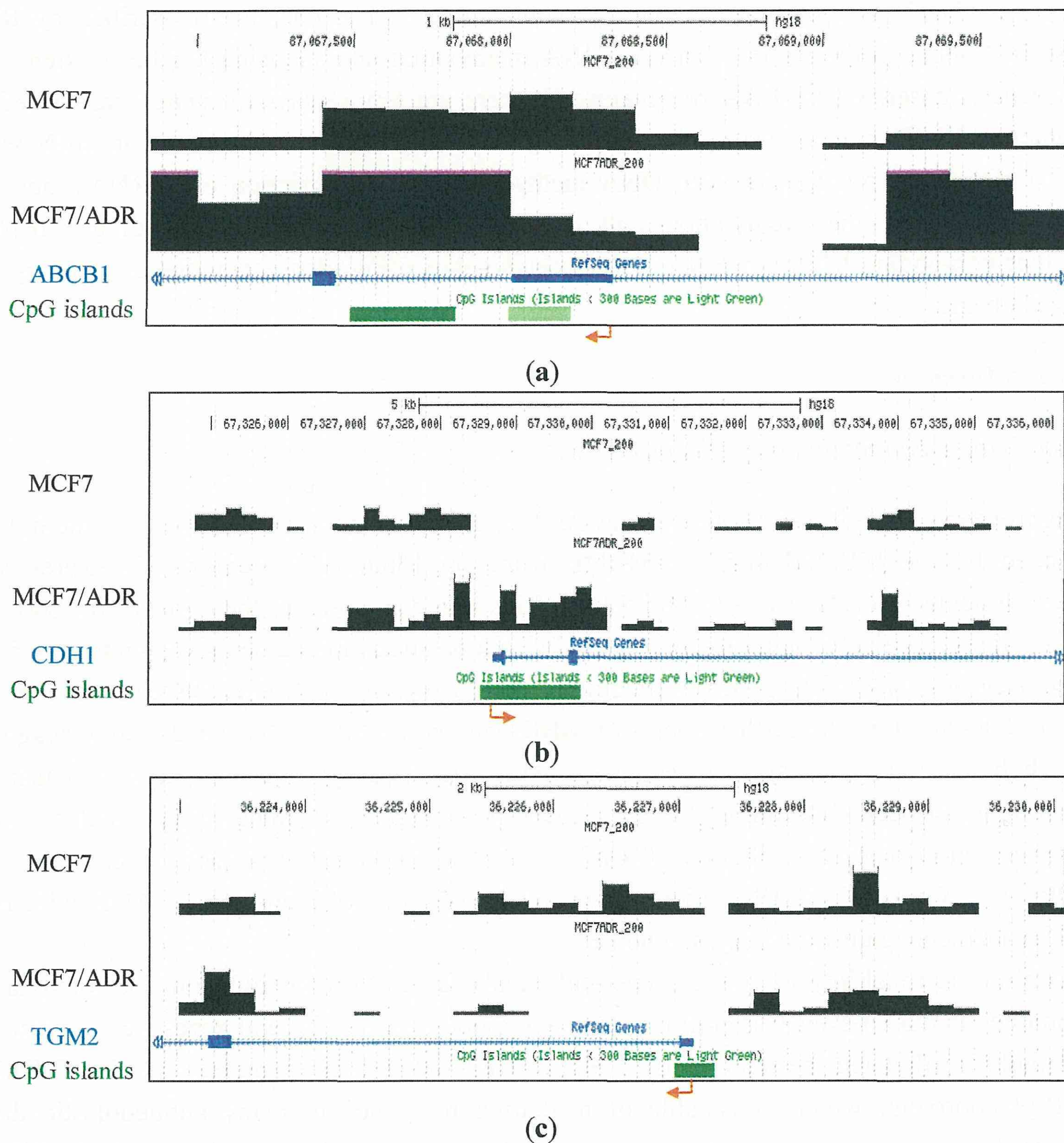
2.1. Genome-Wide Identification of Methylated DNA

To study the genome-wide methylation signature, we performed massive sequencing of methylated DNA enriched by the MBD domain of MBD1, using an Illumina sequencer. We expressed a His-tagged fragment of MBD1 (aa 1–75) in bacteria. This fragment contains the critical MBD domain required for stable and selective binding to methyl-CpG but no structural elements known to contribute to sequence-specific DNA binding [15]. Randomly shared methylated genomic DNA (about 300 bp) was bound to His- and GST-tagged fragments of MBD1 and collected on Dynabeads Talon Magnetic beads, which bind to the His tag. The collected DNA was then purified and sequenced using a next-generation sequencer (Illumina). We performed the genome-wide methylation analysis on a human breast cancer-derived cell line, MCF7, and an adriamycin resistant cancer cell line MCF7/ADR [16]. We obtained 19 million single-end reads for MCF7 and MCF7/ADR with high quality read placement against the human genome.

To determine the reliability of our genome-wide analysis of DNA methylation, the methylation signature attained in our analysis was confirmed using previous reports. Several genes were reported to be differentially methylated between MCF7 and MCF7/ADR. For example, ABCB1 (MDR1), encoding P-glycoprotein, which is capable of mediating resistance to many antineoplastic drugs commonly used to treat breast cancer by acting as an efflux pump, is hypermethylated in MCF7 but demethylated and overexpressed in drug-resistant MCF7/ADR [17]. We confirmed this differential methylation between MCF7 and MCF7/ADR in our analyzed data (Figure 1a). The silencing of CDH1, which encodes E-cadherin, is important in the epithelial-to-mesenchymal transition. It has been reported by others [18] and confirmed by our results that CDH1 is unmethylated in MCF7 but

hypermethylated in MCF7/ADR (Figure 1b). Differential methylation of TGM2 [19], a potential molecular marker for chemotherapeutic drug sensitivity, was also confirmed (Figure 1c).

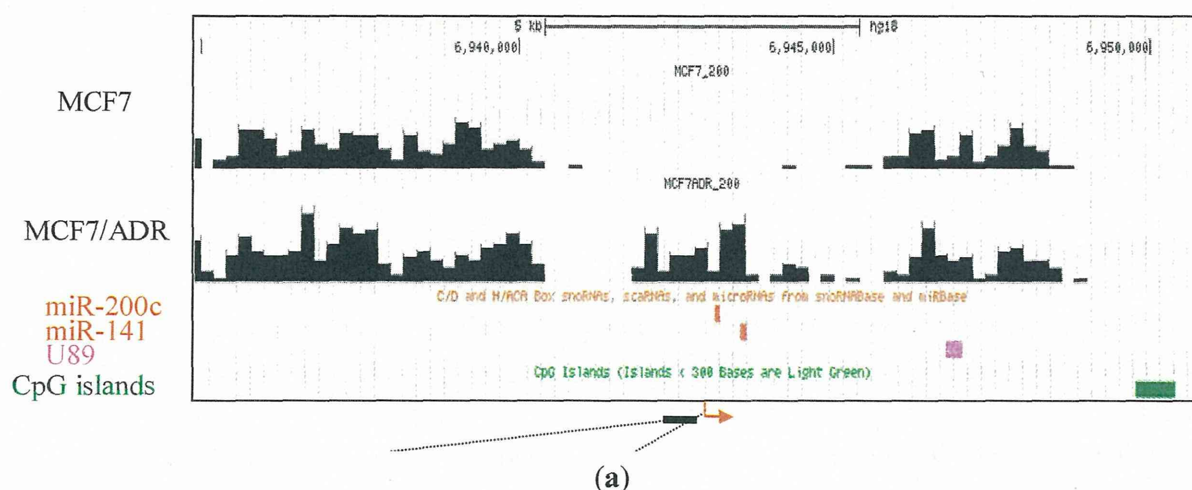
Figure 1. Representative methyl-CpG binding domain (MBD)1DIP-Seq profiles of previously reported differentially methylated promoters between MCF7 and MCF7/adriamycin (ADR). *Y* axis represents the methylation levels of each cell line. Arrows and green bars denote transcription start sites and cytosine-phosphate-guanine dinucleotide (CpG) islands, respectively. (**a**) P-glycoprotein (ABCB1) locus; (**b**) E-cadherin (CDH1) locus; (**c**) transglutaminase 2 gene (TGM2) locus.



Next, we compared the methylation around the miRNA promoters between MCF7 and MCF7/ADR and pick up the differentially methylated regions. By confirming these differentially methylated regions using bisulfite sequencing, we tested the reliability of our analysis. Combining nucleosome mapping with chromatin signatures of promoters, 157 proximal promoters of human miRNA [20] were

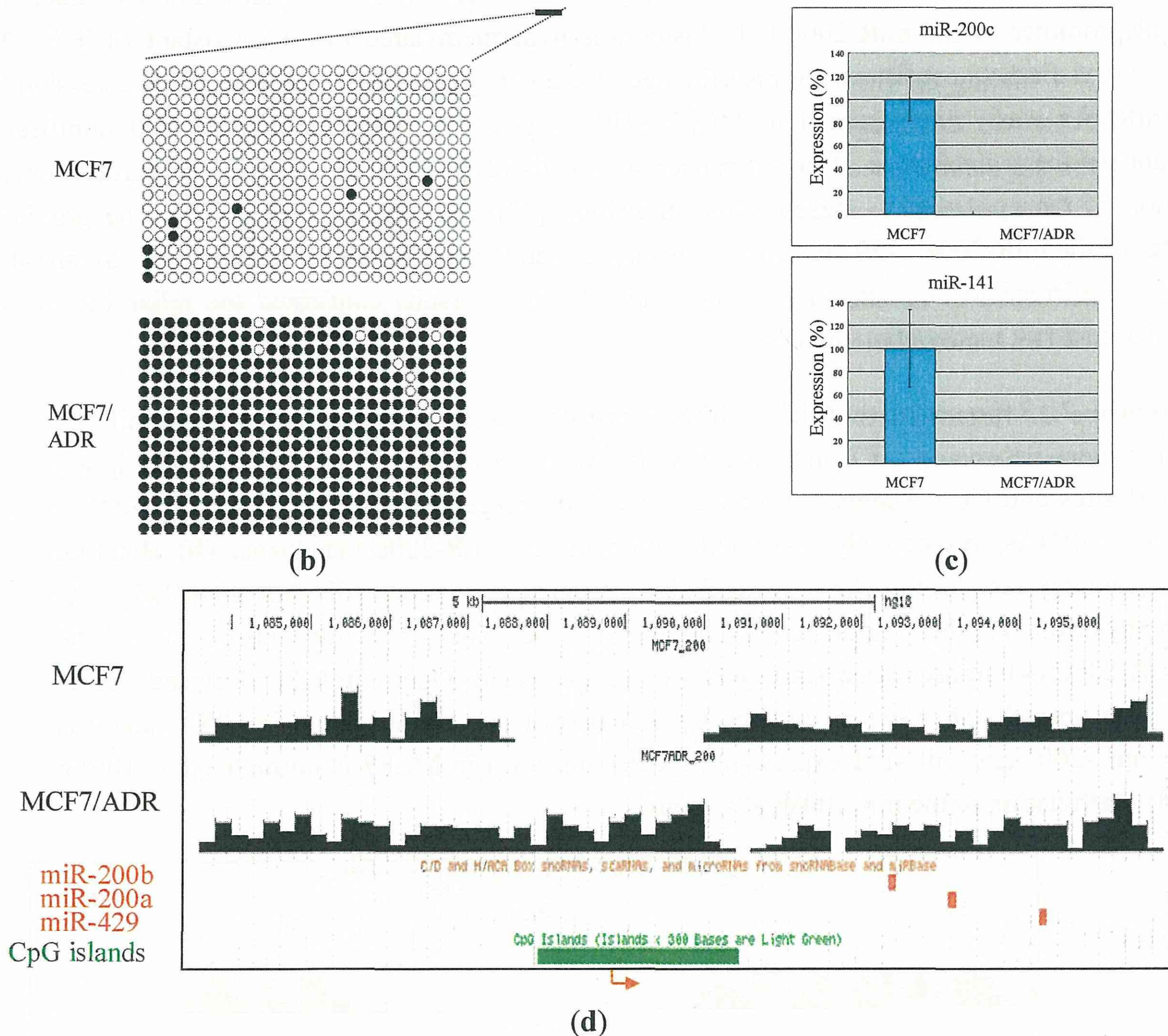
identified and used for the analysis. We found several miRNA clusters, such as miR-200c/141 and miR-200ab/429, which were differentially methylated between these cell lines. For example, the proximal promoter of the miR-200c/141 cluster was hypermethylated in drug-resistant MCF7/ADR (Figure 2a), a finding confirmed by bisulfite sequencing (Figure 2b). In addition, the expression of these miRNAs was downregulated in MCF7/ADR (Figure 2c). Silencing of miR-200 families is important for the maintenance of breast cancer stem cells [21]. This family is also important for the regulation of the epithelial to mesenchymal transition [22] and drug-resistance [23]. The proximal promoter of the miR-200ab/429 cluster was also hypermethylated in MCF7/ADR (Figure 2d), and this was also confirmed to be silenced (data not shown). These results confirmed the reliability of our MBD1-based DNA methylation analysis.

Figure 2. Representative MBD1DIP-Seq profiles of differentially methylated miRNA promoters between MCF7 and MCF7/ADR. Arrows and green bars denote transcription start sites and CpG islands, respectively. Red and magenta blocks indicate pre-miRNAs and scaRNAs, respectively. (**a**) Methylation of the miR-200c/141 locus; (**b**) Bisulfite sequencing results for the differentially methylated promoter of miR-200c/141. The methylation in the region corresponding to the black bar just upstream of the miR-200c/141 transcription start site in (**a**) is presented. Open circles and closed circles denote unmethylated and methylated CpG sites, respectively; (**c**) Real-time-PCR analysis of miR-200c and miR-141 expression. Expression level in MCF7 is normalized as 100%; (**d**) Methylation of the mir-200ab/429 locus.



(**a**)

Figure 2. *Cont.*



2.2. *DNA Methylation* versus *Transcription*

To explore the relationship between gene expression and DNA methylation in the proximal promoter and elsewhere in the gene, we performed microarray analysis using the Agilent platform for miRNA and ordinary genes in MCF7. We were able to use 157 miRNAs, 600 RefSeq non-coding RNAs (ncRNAs) and 235 RefSeq pseudogenes for both expression and methylation analysis. We also used 1000 randomly selected RefSeq protein-coding genes. Before analysis, we plotted the CpG density of each gene category against the position in the genes, since CpG density is an important promoter characteristic. For genes of all categories, the highest CpG density was at the transcription start site, and this is characteristic of promoters (Figure 3). miRNA and protein-coding genes had higher CpG density at the transcription start site compared with other non-coding RNA genes and pseudogenes (Figure 3). We split the genes into two groups: "highly expressed" and "weakly expressed" genes. Highly expressed and weakly expressed genes were defined as those falling within the highest 20% expression quantile and the lowest 20% expression quantile, respectively. For each group, we plotted average cytosine methylation against gene position (Figure 4). This analysis was

performed for each category of gene: miRNA, protein-coding, other non-coding RNA, and pseudogene. In the highly expressed genes, we observed low methylation in the proximal promoters for both miRNAs and protein-coding genes (Figure 4). However, low methylation in the highly expressed genes was not observed in the proximal promoters for the other non-coding RNAs and pseudogenes (Figure 4). Thus, DNA methylation in the proximal promoter of miRNAs is tightly linked to transcriptional silencing, as is the case with protein-coding genes.

Figure 3. CpG density around the transcription start site for each gene category. The average CpG density is plotted against distance from transcription site.

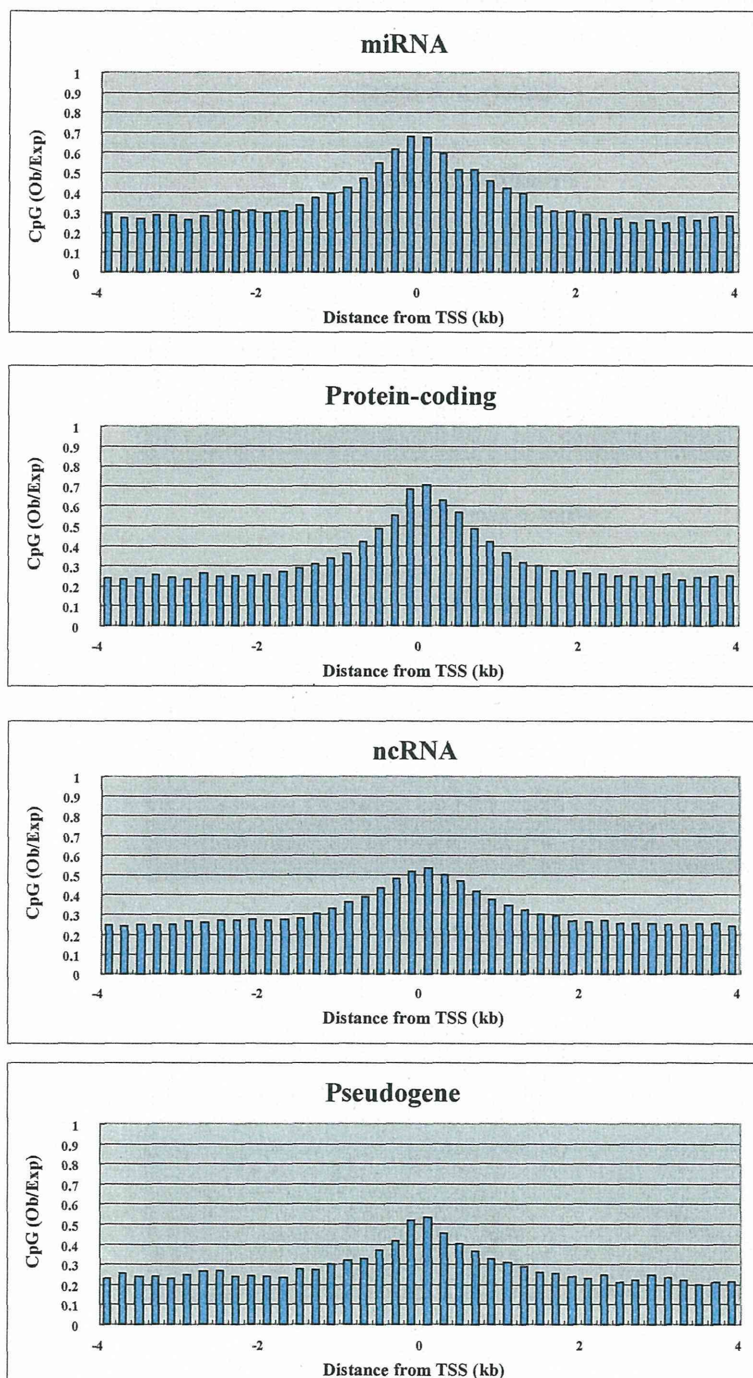
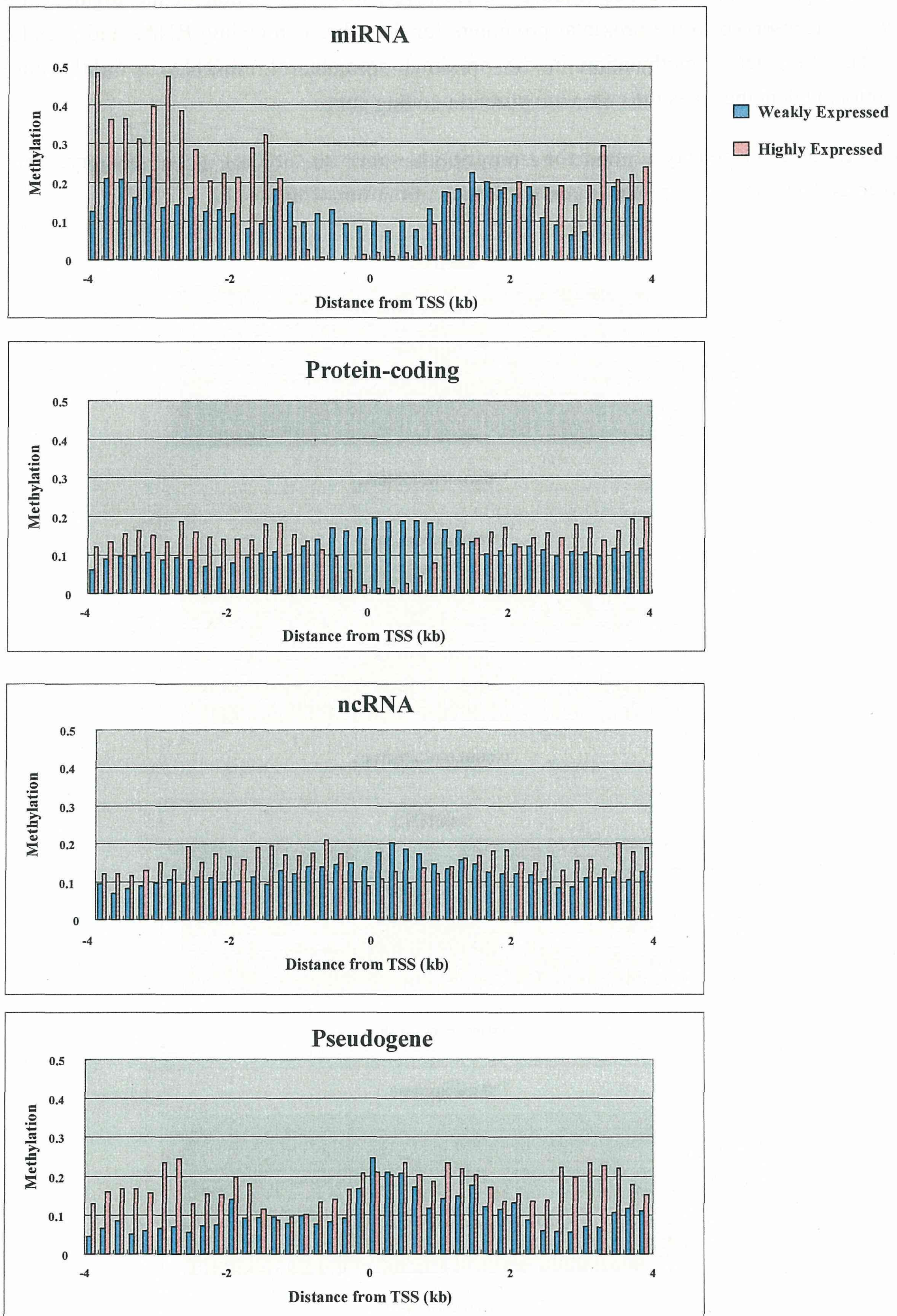


Figure 4. Methylation of weakly expressed and highly expressed genes. The average methylation for each gene category is plotted against distance from transcription start site.



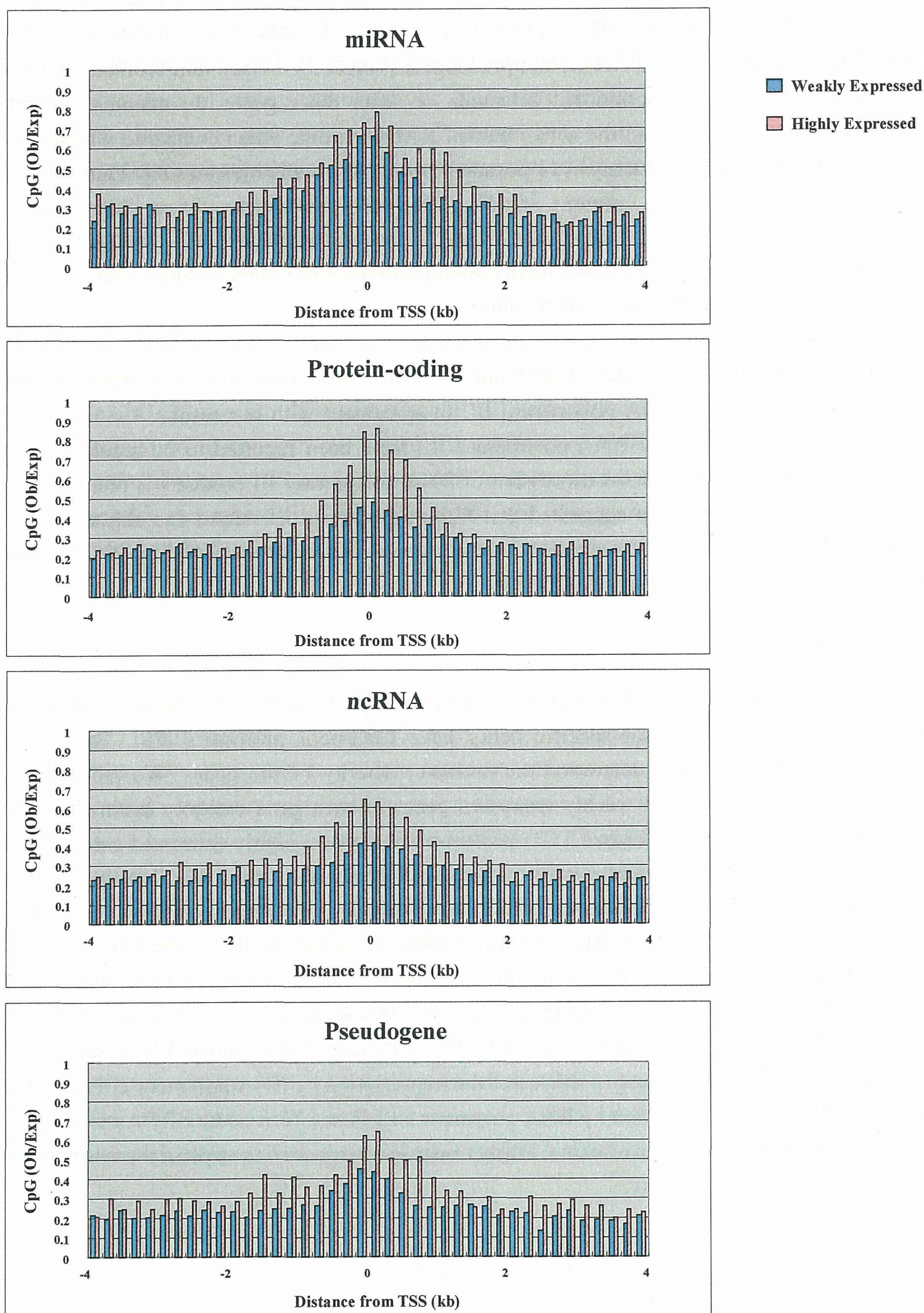
In addition to proximal promoter methylation, the role of non-promoter methylation, such as in enhancers/far-upstream elements and within the body of the gene, was also examined. Methylation was seen in the gene body and far upstream region, with highly expressed genes showing more extensive methylation compared with weakly expressed genes in all categories, including miRNAs, protein-coding, other non-coding RNAs, and pseudogenes (Figure 4). Thus, non-promoter methylation of miRNAs is linked to transcriptional activation, as with other genes. In mammals, gene-body methylation has been observed in the active human X chromosome when compared with its inactive counterpart [24]. Genome-wide analysis of postnatal neural stem cells indicates that Dnmt3a occupies and methylates non-promoter regions flanking proximal promoters of a large cohort of transcriptionally active genes, many of which encode regulators of neurogenesis [25]. Dnmt3a-dependent nonproximal promoter methylation promotes expression of these neurogenic genes by functionally antagonizing Polycomb repression.

Thus, our genome-wide analysis demonstrated for the first time that the relation between DNA methylation and transcription of miRNAs are similar to that of protein-coding genes although some of miRNAs are transcribed by RNA polymerase III. In agreement with our results, U6 snRNA family genes, which are transcribed by RNA polymerase III, have been reported to be regulated by DNA methylation recently [26]. Increased expression of RNA polymerase III products is often observed in transformed cells [27]. It is also suggested that RNA polymerase III output can substantially affect transformation [27]. Therefore, transcriptional regulation of miRNAs transcribed by RNA polymerase III might play an important role in transformation.

2.3. *CpG Density* versus *Transcription*

CpG density underlies many characteristics of promoters. Ubiquitously expressed genes have CpG-rich promoters, while tissue-specific genes have CpG-poor promoters [28]. Therefore, it is possible that CpG density can determine the promoter activity of the genes. We plotted the CpG density of highly expressed and weakly expressed genes in each gene category against the position from the transcription start site (Figure 5). In protein-coding genes, highly expressed genes had higher CpG density in the promoter, while weakly expressed genes had lower CpG density (Figure 5). In other words, ubiquitously expressed genes have stronger promoter activity compared with tissue-specific genes. This characteristic is only weakly observed in gene categories other than protein-coding genes (Figure 5). Therefore, this correlation between CpG density and promoter activity could be related to protein-coding ability. Protein-coding genes are transcribed by RNA polymerase II, while genes coding small non-coding RNAs including some miRNAs are transcribed by RNA polymerase III. It is intriguing to think that the transcription by RNA polymerase II is affected by CpG density, while the transcription by RNA polymerase III is not. Non-coding RNAs including small RNA become important in cancer research. Further research is required to explain the relation between CpG density and promoter activity.

Figure 5. CpG density of weakly expressed and highly expressed genes. The average CpG density of each gene category is plotted against distance from transcription site.



3. Experimental Section

3.1. Cell Lines and Culture Conditions

The human breast cancer-derived cell line, MCF7, and its multi-drug resistant derivative, MCF7/ADR, were cultured in RPMI1640 medium containing 10% FBS.

3.2. Isolation of Methyl-DNA by MBD1

Ten micrograms of genomic DNA were sonicated to 100–500 bp using a Bioruptor UCD-250 (Cosmo Bio, Tokyo) sonicator. The capture reaction was performed by adding 10 mg of sonicated DNA to a mixture of 10 micrograms of His-tagged MBD1 fragment (aa 1–75) and 60 μL of Dynabeads TALON in MBD1 buffer (10 mM Tris, pH 7.5, 160 mM NaCl, 0.1% Tween20) on a rotating mixer, overnight, at 4 °C. The beads were washed four times with MBD1 buffer. The bound methylated DNA was then eluted by digestion with Proteinase K in 50 mM Tris (pH 7.5), 10 mM EDTA, 0.5% SDS, at 50 °C for 3 h. Eluted DNA was purified with MinElute spin columns (QIAGEN).

3.3. Illumina Genome Analyzer Library Preparation and Sequencing

A single-end library was made following the modified manufacturer's protocol with reagents supplied in the Illumina DNA sample kit. Briefly, collected methylated DNA was end-repaired using Klenow and T4 DNA polymerases, phosphorylated with T4 polynucleotide kinase, and adenylated using Klenow exo-DNA polymerase, and oligonucleotide adapters were added using DNA ligase. The ligated product was amplified using adapter-specific primers 1.1 and 2.1 with Phusion DNA polymerase using the following protocol:

98 °C (0:30) + [98 °C (0:10) + 65 °C (0:30) + 72 °C (0:30)] × 10 cycles + 72 °C (5:00)

Amplified DNA was visualized in a 5% Acrylamide TBE gel, and a 300 to 400 bp size range was excised and purified using SPIN-X cartridges (Costar) and MinElute spin columns (QIAGEN). A single-flow cell lane was sequenced in an Illumina Genome Analyzer. The reds were mapped to the reference human genome sequences (hg18). We normalized the tag counts for each region to tags per million (ppm) in a window size of 200 bases, and this value was represented as methylation level.

3.4. Bisulfite Sequencing

One microgram of genomic DNA was bisulfite-treated with the EpiTect Bisulfite kit (QIAGEN) according to the manufacturer's protocol. PCR amplification was performed on bisulfite-modified DNA using the following primers: TTTATGGTAGGAGGATATATTTGTG and CACCTTAAATCAAACAACTTCAAAC. The resulting PCR product was cloned into a pCR2.1 (Invitrogen) TA-cloning vector and was sequenced using a 3130 DNA sequencer (Applied Biosystems).

3.5. Real-Time PCR Analysis of miRNA Expression

Expression of mature miRNAs (miR-200c and miR-141) in human breast cancer cell lines was analyzed by TaqMan miRNA Assay (Applied Biosystems) under conditions defined by the supplier.

3.6. Genome-Wide Gene Expression Analysis

Expression analysis of miRNA and the other genes was carried out using Agilent's microarray-based miRNA and mRNA platforms, respectively. Labeling and hybridization was performed according to the manufacturer's protocol (Agilent Technologies).

4. Conclusions

In this study, we systematically analyzed the methylation and expression of human miRNAs in breast cancer cell lines and compared these levels with those of other gene categories using next-generation sequencing and microarray analysis. We found that DNA methylation in the proximal promoters of miRNAs and protein-coding genes was tightly linked to transcriptional silencing. Expression analysis revealed a correlation between expression level and CpG density of proximal promoters in protein-coding genes; however, this was only weakly observed in miRNA genes. Our observations highlight the epigenetic similarities and differences between miRNA and protein-coding genes.

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