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RESEARCH ARTICLE

UPDATE Correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes [version 2; peer review: 1 approved, 2 not approved]

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Abstract

Background miRNA regulation of target genes and promoter methylation are known to be the primary mechanisms underlying the epigenetic regulation of gene expression. However, how these two processes cooperatively regulate gene expression has not been extensively studied.

Methods Gene expression and promoter methylation profiles of 271 distinct human cell lines were obtained from gene expression omnibus. P-values that describe both miRNA-targeted-gene promoter methylation and miRNA regulation of target genes were computed using the MiRaGE method proposed recently by the author.

Results Significant changes in promoter methylation were associated with miRNA targeting. It was also found that miRNA-targeted-gene promoter hypomethylation was related to differential target gene expression; the genes with miRNA-targeted-gene promoter hypomethylation were downregulated during cell senescence and upregulated during cellular differentiation. Promoter hypomethylation was especially enhanced for genes targeted by miR-548 miRNAs, which are non-conserved, primate-specific miRNAs that are typically expressed at lower levels than the frequently investigated conserved miRNAs.

Conclusions It was found that promoter methylation was affected by miRNA targeting. Furthermore, miRNA-targeted-gene promoter hypomethylation is suggested to facilitate gene regulation by miRNAs that are not strongly expressed (e.g., miR-548 miRNAs).

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UPDATED Changes from Version 1

I have taken a number of steps to address any concerns expressed by the reviewers for the first version of this manuscript.

First of all, I replaced the terminology "miRNA-targeting-specific promoter methylation" with "miRNA-targeted-gene promoter methylation" throughout the manuscript, as suggested.

Second, I emphasized throughout the manuscript that what I found in this research was not a causality, but just a correlation. I clearly denoted that I did not intend to insist that, for example, miRNAs directly induce promoter methylation. I supposed that understanding of the biological mechanisms behind the correlation is not a main topic of this manuscript. Thus, I did not intend to discuss if hypo- and hypermethylation of promoters can be caused by distinct biological mechanisms or not.

Third, I demonstrated that the direct correlation between promoter methylation and gene expression can hardly be a cause of the correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes, by showing that the latter correlation is rather independent of the former correlation (Table 7).

Fourth, I showed that the miRNA-targeted-gene promoter methylation can hardly be an artifact caused by the algorithm employed in MiRaGE, by showing that shuffling gene order destroyed the miRNA-targeted-gene promoter methylation (Table 4).

Fifth, I added the sentence that apparent regulation of target genes by miR-548 miRNAs can also be mediated by unknown factors other than promoter methylation.

Sixth, I corrected the description of MiRaGE as suggested.

Seventh, I compared promoter methylation between miRNA-target genes and miRNA-non-target genes and found significant differences (Table 3).

See referee reports

Introduction

The epigenetic regulation of gene expression¹ has recently attracted the interests of many researchers. Epigenetic modifications regulate gene expression without modifying DNA sequences. Examples include promoter methylation², histone modification³, the binding of transcription factors to gene promoter regions⁴, and miRNA regulation of target genes⁵.

Promoter methylation and miRNA regulation of target genes are particularly important in the epigenetic regulation of gene expression. Promoter methylation is relatively stable, long term, and in some cases, heritable. It is generally believed that genes with hypermethylated promoters are repressed. In addition, there is mounting evidence that DNA methylation is involved in the development and progression of certain disease states. For example, aberrant methylation in cancer is frequently observed⁶ and the distinct patterns of promoter methylation between monozygotic (MZ) twin pairs have also been found to result in different health conditions⁷. In contrast to DNA methylation, miRNA regulation of target genes is more flexible and can change even during cellular differentiation⁸. miRNA expression is often tissue-specific, and similar to DNA methylation, miRNA expression has been linked to human disease⁹.

Thus, although miRNA-directed gene regulation is thought to result in subtle changes, it is generally believed that miRNAs are involved in many important biological processes ranging from cell division to aging.

Although DNA methylation of miRNA promoters has been studied extensively (e.g., with respect to tumor formation¹⁰), the relationship between promoter methylation and miRNA regulation of target genes has not been thoroughly investigated. One likely reason for this is that the regulation of gene expression by promoter methylation is a form of pre-transcriptional control, whereas miRNA regulation of target genes is a form of post-transcriptional control, with the former taking place inside the nucleus and the latter outside the nucleus (cytoplasm). Thus, these two mechanisms are separated by both time and space, and as a result, there have not been plausible biological reasons to suspect that promoter methylation and miRNA-mediated gene regulation operate in concert.

However, Su *et al.*¹¹ recently found that miRNAs have a tendency to target genes with hypomethylated promoters. To my knowledge, their study was the first report suggesting coregulation of gene expression by promoter methylation and miRNAs. In addition, Sinha *et al.* reported that genes promoters with high CpG content were more often targeted by miRNAs¹². Saito and Sætrom also discussed the relationships between miRNA-mediated gene regulation and various features of target genes, but they did not consider the methylation status of target gene promoters¹³. Although the study of Su *et al.* represents the first evidence of a direct link between promoter methylation and miRNA regulation, the biological significance of their findings is not clear. In this study, I report that promoter methylation is associated with miRNA-targeting; that is, the amount of methylation observed at a given gene promoter is dependent on whether that gene is also a target of miRNA regulation. Furthermore, miRNA-targeted-gene promoter methylation is also related to how miRNAs regulate target gene expression. In particular, I reveal that miR-548 miRNAs target genes are associated with highly hypomethylated promoters.

Methods

Promoter methylation profiles

In this study, I used publically available promoter methylation profiles from various resources obtained from GEO ID: GSE30653¹⁴. This included 283 human promoter methylation profiles for distinct cell lines, ranging from hESC to various somatic samples, measured using the HumanMethylation27 BeadChip (Illumina), which provides an efficient approach for surveying genome-wide DNA methylation profiles. The HumanMethylation27 panel targets CpG sites located within proximal promoter regions of transcription start sites (TSS). Thus, it was suitable for the purpose of this study. Promoter methylation profiles (GEO ID: GSE30653) also included data from both IMR90 and MRC5 cell lines, which were used to

investigate relationships between promoter methylation and previously reported miRNA regulation and miRNA expression profile data^{15,16}. Promoter methylation profiles in both BG02 and BG03 were also included in this study, and were compared to miRNA regulation and miRNA expression profile data (see below).

Additional promoter methylation profiles in IMR90 cell lines were obtained from GEO IDs, GSM868008¹⁴, GSM739940¹⁷, and GSM375442¹⁸. They were compared to IMR90 promoter methylation profiles (GEO ID: GSM760387 within GSE30653). GSM868008 was included in the GEO ID, GSE31848, which were generated using the Illumina HumanMethylation450 BeadChip. This BeadChip allowed us to interrogate > 485000 methylation sites per sample at single-nucleotide resolution. In addition, because this array also includes CpG sites outside of promoter regions, I restricted probes to a subset labeled as either TSS200 or TSS1500. Data from GSM739940 includes IMR90 promoter methylation profiles measured by the Illumina HumanMethylation27 BeadChip. However, because these data were generated by a different research group than that of GSE30653, I tested profiles from this dataset to confirm that obtained results were not research group dependent. Finally, I also used methylation profile data from GSM375442, which were generated using next generation sequencing (NGS). CpG methylation profiles from promoter regions were extracted using Bismark Software (Ver. 0.7.4)¹⁹ (see below); promoter regions were defined as nucleotide positions between -200 and +1200 basepairs (bp) from transcription start sites (TSSs).

mRNA and miRNA expression profiles

In order to compare miRNA-targeted-gene promoter methylation with target gene miRNA regulation and miRNA expression profile data from BG02 and BG03 cell lines, both miRNA and mRNA profiles were obtained from GEO ID: GSE14473²⁰. Gene (mRNA) expression profiles of undifferentiated and differentiated BG02 and BG03 cell lines were obtained from the GEO IDs, GSM551204 and GSM551206, and GSM551216 and GSM551218, respectively. Corresponding miRNA expression profiles of these two cell lines were obtained from the GEO IDs, GSM361147 and GSM361271 (BG02) and GSM361288 and GSM361289 (BG03). Raw data files were downloaded for further analysis and were normalized so as to have a mean of 0 and a variance of 1.

Investigation of miRNA-targeted-gene promoter methylation

In order to infer miRNA-targeted-gene promoter methylation, I employed the MiRaGE method²¹ (see below). The MiRaGE method, which was implemented on a [public domain MiRaGE server](#) and [Bioconductor MiRaGE package](#), was first used to infer the contribution of miRNA to the measured expression levels. This software was designed to accept

expression profiles of the target genes in question; however, in this study, I used this method to infer miRNA-targeted-gene promoter methylation by substituting gene expression profiles with the promoter methylation profiles of each gene.

I first prepared a control dataset (pseudo) in which the expression level of all genes assigned a value of 1. Then, the amount of methylation at each gene was used as values of treatment data set. Although, the ratio of the number of methylated sites to the total number of methylated and non-methylated sites is typically used to describe promoter methylation levels, I employed a method in which total methylation values were used. I used this approach because I found that *P*-values computed when using methylation data were more strongly correlated to the *P*-values calculated from target gene miRNA regulation data (see below), which is likely due to the fact that the frequency of CpGs is also related to miRNA targeting¹²; i.e., genes with promoters that contain more CpGs were more often targeted by miRNAs as mentioned above. Using this procedure, I attributed two *P*-values to each miRNA, one expressing the degree of promoter hypermethylation, and the other expressing the degree of promoter hypomethylation. The approach used to compute *P*-values representing promoter methylation are described below for each of the different methodologies and/or datasets used.

GSM868008

Promoter methylation profiles used to replace “gene expression” values within the MiRaGE method were

$$\begin{aligned}x_{\text{control},i} &= 1, \text{ (pseudo) control set} \\x_{\text{treat},i} &= \exp(M_{0i}), \text{ treatment set}\end{aligned}$$

where M_{0i} represented the scaled values of signal_B (intensity estimated of methylated DNA), which were expressed as the amount of promoter methylation of *i*th gene,

$$\begin{aligned}M_{0i} &\equiv \frac{M_i - \langle M_i \rangle}{\sigma_{M_i}} \\ \langle M_i \rangle &\equiv \frac{1}{N} \sum_i M_i \\ \sigma_{M_i} &\equiv \sqrt{\frac{1}{N} \sum_i (M_i - \langle M_i \rangle)^2},\end{aligned}$$

where N was the total number of genes considered and M_i was the raw value of signal_B. This signified that the amount of promoter methylation was scaled so as to have a mean $\langle M_{0i} \rangle$ of 0 and a standard deviation $\sigma_{M_{0i}}$ of 1. $\exp$ was applied in this instance because I wanted to consider the amount of methylation rather than the ratio of methylation. Because *P*-values were computed after the pair of input values were transformed to a logarithmic ratio, substituting 1 in the control dataset and an

exponential value in the treatment dataset based on raw values resulted in the usage of raw values of differential expression/promoter methylation (see below).

GSE30653

Promoter methylation profiles used to replace “gene expression” values within the MiRaGE method were

$$\begin{aligned} x_{\text{control},i} &= 1, \text{ (pseudo) control set} \\ x_{\text{treat},i} &= M_i + C_i, \text{ treatment set} \end{aligned}$$

where C_i took on the value of 1 only when $M_i = 0$; otherwise, it took on the value 0, so as to avoid infinite values after transformation to the logarithmic ratio.

GSM739940

Promoter methylation profiles used to replace “gene expression” values within the MiRaGE method were

$$\begin{aligned} x_{\text{control},i} &= 1, \text{ (pseudo) control set} \\ x_{\text{treat},i} &= \exp(\beta_i), \text{ treatment set} \end{aligned}$$

where β_i was the ratio of methylated sites to unmethylated sites,

$$\beta_i \equiv \frac{M_i + C}{M_i + U_i + C},$$

where U_i was the signal from unmethylated sites (signal_A) and C was the regulation constant, which typically took the value of 100. Since only β values were deposited in the public datasets used, their use could not be avoided; however, as a result, the correlation with target gene miRNA regulation was substantially decreased. An explanation for this is noted above.

GSM375442

Promoter methylation profiles used to replace “gene expression” values within the MiRaGE method were

$$\begin{aligned} x_{\text{control},i} &= 1, \text{ (pseudo) control set} \\ x_{\text{treat},i} &= \exp\left(\frac{M_i}{\max(M_i)}\right), \text{ treatment set} \end{aligned}$$

where $\max(M_i)$ was the maximum value of M_i and M_i was computed in this case as follows:

$$M_i \equiv \sum_{j \in \text{promoter of gene } i} y_j,$$

where y_j , $0 \leq y_j \leq 100$ was the percentage of methylation at site j , which was computed using the Bismark Software¹⁹ (see below). The summation was taken over the length of the promoter region as defined above (i.e., between -200 bp and +1200 bp from the TSS).

Methylation computation by Bismark Software

The following command line inputs were used to generate methylation values of CpG sites within the Bismark Software package¹⁹.

```
% bismark_genome_preparation \
--path_to_bowtie bowtie_dir \
--verbose ./hg19/ &
% R
>x <- scan("GSM375442_CpgMIP-IMR90.seq.txt",
sep="\n", what=character(0))
>write.table(file="sequence.fa",
paste(paste(">p", 1:length(x), sep=" "),
x, sep="\n"), sep="\n", row.names=F,
quote=F, col.names=F)
>q()
% bismark ./hg19/ \
--path_to_bowtie bowtie_dir \
--bowtie2 -f sequence.fa
% methylation_extractor -s --comprehensive \
sequence.fa_bt2_bismark.sam
% genome_methylation_bismark2bedGraph_v3.pl \
CpG_content_sequence.fa_bt2_bismark.sam.txt \
> sequence.fa_bt2_bismark.sam.bed
```

where `bowtie_dir` is the directory where `bowtie2` is installed. In this study R²² was used, but scripts executed by R can also be performed by any other alternative languages. `GSM375442_CpgMIP-IMR90.seq.txt` is a file downloaded from GEO and `sequence.fa_bt2_bismark.sam.bed` includes methylation percentages of each CpG site, y_j , which was explained above.

Inference of miRNA-targeted-gene promoter methylation/miRNA regulation of target genes using the MiRaGE method

The inference of miRNA-targeted-gene promoter methylation/miRNA regulation of target genes was carried out using the MiRaGE method, which has been described previously²¹.

Although the MiRaGE method is typically used for datasets with two experimental conditions, each of which contains more than one replicate, I used this method for instances in which each condition consisted of only a single replicate. In each case, I based the analysis on the premise that for a given gene i , there were a pair of gene expression datasets or promoter methylation profile datasets, which were measured under a control condition $x_{\text{control},i}$ and a treatment condition $x_{\text{treat},i}$. From this, I computed the logarithmic ratio

$$\Delta x_i \equiv \log\left(\frac{x_{\text{treat},i}}{x_{\text{control},i}}\right)$$

In such an example, when a difference between raw values is favorable, exponential values $\exp(x_i)$ can be used instead of x_i , in which case, I got

$$\Delta x_i = \log \left\{ \frac{\exp(x_{\text{treat},i})}{\exp(x_{\text{control},i})} \right\} = x_{\text{treat},i} - x_{\text{control},i}$$

When computing P -values that rejected the null hypothesis, by using the alternative hypothesis that Δx_i s of the target genes of the miRNA m are less (greater) than those that are off-target but form a target of other miRNAs, I computed

$$P_m^{<(>)} \equiv P[\{\Delta x_i \mid i \in G_m\} < (>) \{\Delta x_i \mid i \in G'_m\}]$$

where $P[A < (>) B]$ represented P -values computed by statistical tests when sets A and B are compared. The tests implemented within the MiRaGE Server/package are the t -test, Wilcoxon rank sum test, and Kolmogorov-Smirnov test.

Thus, determining whether $A < (>) B$ depends on the selected statistical test used. I used G_m to represent the set of genes targeted by miRNA m and G'_m as the intersection of the set of off-target genes of miRNA m and the set of target genes of all other miRNAs. It should be noted that genes that were not the targets of any miRNAs were totally excluded from the analysis; however, all miRNAs were considered and no exclusions based on conservation were applied. When inferring promoter methylation, $x_{\text{control},i} = 1$ and $x_{\text{treat},i}$ were used to represent the amount of promoter methylation. When inferring miRNA regulation of target genes during cell senescence in IMR90 and MRC5 cell lines, $x_{\text{control},i}$ was used to represent gene expression of young cell lines and $x_{\text{treat},i}$ was used to represent gene expression in senescent cell lines. When inferring regulation of target genes during differentiation in BG02 and BG03 cell lines, $x_{\text{control},i}$ was used to represent gene expression in undifferentiated cell lines and $x_{\text{treat},i}$ for gene expression in differentiated cell lines.

Correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes

I used two types of P -values, $P_{m:\text{methy}}^{<(>)}$ which corresponded to the miRNA-targeted-gene promoter methylation, and $P_{m:\text{regul}}^{<(>)}$ which corresponded to the miRNA regulation of target genes of miRNA m . When $P_{m:\text{methy}[\text{regul}]}^{<(>)}$ was small enough, the target genes of miRNA m are significantly hypermethylated or hypomethylated and thus downregulated or upregulated. In order to see if these two types of P -values were correlated, I computed various correlation coefficients:

$$\begin{aligned} \rho^{\text{Pearson}} &\equiv \rho[P_{m:\text{methy}}, P_{m:\text{regul}}] \\ \rho_{\log}^{\text{Pearson}} &\equiv \rho[\log(P_{m:\text{methy}}), \log(P_{m:\text{regul}})] \\ \rho^{\text{Spearman}} &\equiv \rho[\text{rank}(P_{m:\text{methy}}), \text{rank}(P_{m:\text{regul}})] \end{aligned}$$

and accompanied P -values to reject null hypothesis that $\rho = 0$ by using the alternative hypothesis that $\rho \neq 0$. I used $\rho[a, b]$ as the Pearson's correlation coefficients between a and b and $\text{rank}(x_m)$ as the rank order of x_m among $\{x_m\}$. $P_{m:z}$ where $z \in \{\text{methy}, \text{regul}\}$ was either $P_m^<$, $P_m^>$, $1 - P_m^<$ or $1 - P_m^>$. Thus, there were $4 \times 4 = 16$ possible combinations of $P_{m:\text{methy}}$ and $P_{m:\text{regul}}$. ρ s of the Kolmogorov-Smirnov test and $\rho_{\log}^{\text{Pearson}}$ s for all tests changed when $P^{<(>)}$ was replaced with $1 - P^{>(<)}$ because $P^{<(>)} \neq 1 - P^{>(<)}$ for the Kolmogorov-Smirnov test and $\log(P^{<(>)}) \neq \log(1 - P^{>(<)})$ for all tests. Thus, optimal combinations of the maximum absolute correlation coefficients were employed.

The reciprocal relationship between miRNA regulation of target genes and miRNA expression during differentiation in BG02 and BG03

In contrast to the cell senescence study¹⁵ in which miRNA expression was investigated by NGS, only microarray measurements were available for differentiation in BG02 and BG03 cell lines. Due to issues related to the accuracy and quality of microarray data and the relatively small amount of miRNA expression, very few miRNAs were found to be differentially expressed between undifferentiated and differentiated cell lines. Thus, miRNAs that were differentially expressed between undifferentiated and differentiated cell lines were selected based on two criteria before being subjected to further analyses:

- Absolute differential expression $|x_{\text{diff},i} - x_{\text{undiff},i}| > \Delta x_c$, where $x_{\text{diff},i}$ and $x_{\text{undiff},i}$ were the normalized expression of gene i of differentiated and undifferentiated cell lines, respectively. Δx_c was set as the threshold value that could be used to select genes associated with significant differential expression during differentiation.
- For this method, it was required that adjusted P -values based on the BH criterion²³ were less than 0.05 for significant up-regulation or downregulation. Here, P -values were computed using a t test between two sets of probe values attributed to gene i .

After Δx_c was suitably selected, the correlation coefficient between differential expression $x_{\text{diff},i} - x_{\text{undiff},i}$ and $\log(P_{m:\text{regul}}^<)$ were computed. Positive values were taken to indicate a reciprocal relationship between miRNA expression and miRNA regulation of target genes; smaller $P_{m:\text{regul}}^<$ were assumed to signify that target genes were upregulated (and vice versa).

Thus, a reciprocal relationship required that miRNA were downregulated, i.e., $x_{\text{diff},i} - x_{\text{undiff},i} <(>)0$, which should result in a positive correlation between $x_{\text{diff},i} - x_{\text{undiff},i}$ and $\log(P_{m:\text{regul}}^<)$. Since miRNA names in GSE14473 were old (miRBase, release 9.1), they were converted to miRNA names used in the present version of the MiRaGE software package (miRBase release 18) and by the miRConverter implemented in miRSystem²⁴.

Ranking of miRNAs having target genes with miRNA-targeted-hypomethylated promoters

miRNAs were ranked based on $P_m^>$ in each of the 283 samples in GSE30653, after excluding 12 control samples. Each miRNA was ranked within each sample, and then, the cumulative rank was determined for all 270 samples.

Ranking of miRNA regulation of target genes

miRNAs were ranked based on P -values for either downregulation during cell senescence (IMR90 and MRC5) or upregulation during differentiation (BG02 and BG03) for each statistical test. Then, each miRNA was ranked based on the order summed up over three statistical tests.

Comparison of promoter methylation between miRNA-target genes and miRNA-non-target genes

I compared promoter methylation between miRNA-target genes and miRNA-non-target genes for each of the 270 samples in GSE30653, excluding 12 control samples. Statistical tests used were the two-sided t test, Wilcoxon rank sum test, and Kolmogorov-Smirnov test, resulting in 270 P -values for each test.

P -value computation for miRNA-targeted-gene promoter methylation after random permutation

In order to see if P -values for miRNA-targeted-gene promoter methylation, $P_m^>$, changed after the random gene permutation, gene IDs were randomly permuted for each of the 270 samples. Then, the number of significant miRNAs whose target gene promoter was significantly hypomethylated (P -values computed with t test and corrected using the BH criterion²³ were less than 0.05) was counted. The numbers were averaged over one hundred independent random permutations.

Significance of correlation between miRNA-targeted-gene promoter methylation and miRNA-mediated regulation of target genes after random gene permutation

In order to see if the correlation between miRNA-targeted-gene promoter methylation and miRNA-mediated regulation of target genes changed after random gene permutation while conserving the correlation between promoter methylation and gene expression, promoter methylation and gene expression profiles needed to be permuted simultaneously. However, because gene expression and promoter methylation were measured by distinct microarrays, the number of probes attributed to each gene in some cases differed between the microarray

used for gene expression measurement and promoter methylation. Also, multiple CpG sites may be attributed to each gene, while gene expression is unique to each gene. In order to overcome this difficulty, multiple probes or CpG sites were first grouped based on overlapping RefSeq mRNA IDs. RefSeq gene IDs were then shuffled and P -values were computed using MiRaGE, which employs a t test for both miRNA-targeted-gene promoter methylation and miRNA-mediated regulation of target genes, before and after random permutation. The methylation data sets used were GSM868008 (for IMR90 cell line) and GSE30653 (for MRC5, BG02 and BG03). ρ^{Pearson} were computed with $P_{m:\text{methy}}^< = P_m^<$ and $P_{m:\text{regul}}^< = P_m^<$. Gene expression profiles reported previously for both IMR90 cell lines and MRC5 cell lines were used¹⁵. The maximum and minimum values of ρ^{Pearson} s among 100 independent random permutations are presented. The geometric average for over 100 random permutation of P -values attributed to ρ^{Pearson} was also computed. In addition, P -values associated with ρ^{Pearson} before random permutation was estimated based on 100 random permutation ensembles assuming a normal distribution. The normality of the distribution of correlation coefficients from 100 random permutations was checked using both the Shapiro-Wilk normality test and the Kolmogorov-Smirnov test (no significant differences from a normal distribution were detected).

Results and discussion

Significant promoter hypomethylation of miRNA target genes

Based on the inference of data produced using the MiRaGE method²¹ (see Methods), the promoters of genes that are targets of 70–90% of human miRNAs were significantly hypomethylated, dependent on the statistical tests used, and the definition of promoter methylation levels: the β -value or the amount of methylation (see Table 1 and Additional files). This finding was consistent with conclusions made by Su *et al.*¹¹, who stated that miRNAs had a tendency to target genes with hypomethylated promoters. Given that promoter methylation patterns do not change drastically between certain cell types, the amount of miRNA-targeted-gene promoter methylation is also highly cell-type-independent. Mean correlation coefficients of $P_m^>$ ranged between 0.8 and 0.9, again depending on the statistical test used and the definition of promoter methylation levels (Table 2).

In order to confirm that the observed miRNA-targeted-gene promoter methylation was really caused by miRNA targeting, I compared promoter methylation of miRNA target genes with that of miRNA non-target genes. In order to check this point, I have computed P -values that compare promoter methylation between miRNA target genes and miRNA non-target genes (Table 3). I found that the difference was strongly significant. P -values were clearly small enough to be significant even if any multiple comparison corrections were taken into account. This significant difference observed here is coincident with the findings of Su *et al.*¹¹ and supports the argument that the observed

Table 1 miRNA-targeted-gene promoter hypomethylation.

Amount of methylation			
Statistical tests	Number of miRNAs		
	Not significant	Significant	
t-test	136054	384537	74%
Wilcoxon rank sum test	144572	376019	72%
Kolmogorov-Smirnov test	129998	390593	75%
β -values			
Statistical tests	Number of genes		
	Not significant	Significant	
t-test	39390	481201	92%
Wilcoxon rank sum test	45188	475403	91%
Kolmogorov-Smirnov test	41952	478639	92%

The summation of the number of miRNAs that target genes with significantly hypomethylated promoters in each cell line. The total numbers of miRNAs and cell lines were 1921 and 270, respectively (the number of cell lines was out of 283 in GSE30653, excluding 12 control samples). Thus, the total number of combinations of an miRNA and a cell line is $1921 \times 271 = 520591$. Promoter methylation levels were defined as either the amount of methylation or the β -values. Observed P -values based on the BH criterion²³, P_{adj} of < 0.05 were used to signify the promoters of the genes targeted by miRNAs that significantly hypomethylated. P -values were adjusted using the p.adjust function in R²². A complete list of raw (not adjusted) P -values is available in additional files 1 to 6. Cell line names can be also identified as column names of the additional file 1 to 6.

Table 2 Mean correlation coefficients between P -values of miRNA-targeted-gene promoter hypomethylation of samples. Mean Pearson's correlation coefficients of P_m^2 between pairs of cell lines.

Statistical test	Averaged correlation coefficients
Amount of methylation	
t-test	0.9112043
Wilcoxon rank sum test	0.8981256
Kolmogorov-Smirnov test	0.8263153
β -values	
t-test	0.8919359
Wilcoxon rank sum test	0.8832241
Kolmogorov-Smirnov test	0.8257556

miRNA-targeted-gene promoter methylation was really mediated through miRNA targeting.

It may seem strange that I did not investigate promoter hypermethylation and hypomethylation distinctly, because these two should have different biological mechanisms. However, there are some cases that miRNA-mediated regulation of target genes is known to function toward opposite direction when third players take place. For example, some miRNAs can remove Ago proteins

that bind other miRNAs²⁵. In such cases, target genes of these miRNAs are assumed to be upregulated, as miRNAs are generally believed to suppress the expression of their target genes. miRNA sponges can also inhibit miRNA suppression of target genes²⁶. miRNA sponges absorb miRNAs that bind to the RNA-induced silencing complex (RISC). Because of this effect, the expression of miRNAs targets tends to increase with increasing concentrations of miRNA sponges. The biological mechanisms that mediate the interactions between miRNA and methylated promoters, and the role of third players are unknown, thus, it is not always necessary to distinguish the mechanisms underlying the hyper- and hypomethylation of miRNA target genes.

Another possible issue is that miRNA-targeted-gene promoter methylation may represent an artefact caused by the complex algorithm employed by MiRaGE. In order to deny this possibility, I computed miRNA-targeted-gene promoter methylation after random permutation (Table 4), from which I determined that there was no significant miRNA-targeted-gene promoter methylation. Thus, it is apparent that random permutation destroys miRNA-targeted-gene promoter methylation completely. This demonstrates that miRNA-targeted-gene promoter methylation is not an artifact but a real outcome.

Significant correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation

In order to see if miRNA-targeted-gene promoter methylation was genuinely related to the miRNA regulation of target genes, I compared P -values of miRNA-targeted-gene promoter methylation to P -values of miRNA regulation of target genes during the senescence of IMR90 and MRC5 cell lines^{15,16} and during the differentiation of BG02 and BG03 cell lines²⁰. It was clear that promoter methylation and miRNA regulation of target genes were significantly correlated during both cell senescence and differentiation (Table 5), despite the fact that correlation coefficients exhibited opposite directionalities in cell senescence and differentiation. This means that genes with miRNA-targeted-gene promoter hypomethylation are downregulated during cell senescence, but upregulated during differentiation.

To confirm that the significant correlation noted above between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes was not a false-positive finding, I also tested for this association in IMR90 cell lines by using a different microarray and NGS approach, as described previously¹⁸ (Table 6). Again, I observed a significant correlation between promoter methylation status and miRNA regulation, suggesting that the relationship found between these two mechanisms was independent of experimental condition.

However, these findings may not represent a true correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation. For example, if

Table 3 *P*-values that represent the difference of promoter methylation between miRNA target genes and miRNA non-target genes.

<i>t</i> test		Wilcoxon rank sum test		Kormogov-Smirnov test	
Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
1.38×10^{-60}	4.66×10^{-25}	4.26×10^{-105}	1.56×10^{-50}	0	0

The minimum and maximum values are among 270 cell line samples. 0s for Kolmogorov-Smirnov test mean that they cannot be distinct from 0 within numerical accuracy.

Table 4 miRNA-targeted-gene promoter hypomethylation after random gene permutation.

Amount of methylation			
Statistical tests	Number of miRNAs		
	Not significant	Significant	
t-test	520585	6	1.1 x 10 ⁻³ %
Wilcoxon rank sum test	520588	3	5.8 x 10 ⁻⁴ %
Kolmogorov-Smirnov test	520585	6	1.1 x 10 ⁻³ %

The summation of the number of miRNAs that target genes with significantly hypomethylated promoters in each cell line after random permutation. See Table 1 for other notations. The values are averaged over 100 random permutation ensembles.

the observed correlation is genuine, then the correlation between gene expression and promoter methylation should differ between miRNA target genes and non-target genes. Alternatively, a correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation could simply be a bi-product of the well-documented direct correlation between gene expression and promoter methylation. These issues arise from two assumptions: (1) the correlation between gene expression and promoter methylation is strong enough to mediate a correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation; and (2) the correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation should be strongly related to, or even directly caused by, the direct correlation between gene expression and promoter methylation. However, these two assumptions do not necessarily need to be true in all cases. For example, the correlation between gene expression and promoter methylation is not always strong and the correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation could be highly significant in instances in which the relationship between gene expression and promoter methylation is weak.

In order to test the first assumption, I computed the correlation between gene expression and promoter methylation, and revealed that the correlation between gene expression and promoter methylation is much weaker than the correlation between miRNA-mediated regulation of target genes and

miRNA-targeted-gene promoter methylation (see the column entitled “gene expression vs promoter methylation” in Table 7). This suggests that the correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation is not likely the outcome of the correlation between gene expression and promoter methylation, but possibly vice versa. Next, I considered the second assumption, which also need not always be true. From a mathematical point of view, a correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation does not have to be directly related to the correlation between gene expression and promoter methylation. The reason is as follows. It is always possible to shuffle gene order with conserving the correlation between gene expression and promoter methylation, because the correlation does not change between pre- and post-permutation if pairs of promoter methylation and gene expression can be conserved. However, permutation inevitably alters both miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation, because permutation changes the set of genes targeted by each miRNA.

Suppose we have N genes and M miRNAs. Even if we permute gene id g in both microarrays that measure gene expression and promoter methylation simultaneously, the correlation between gene expression and promoter methylation does not change, because pairs of gene expression and promoter methylation are conserved. However, G_m and G'_m are always altered, as permutation inevitably removes some genes from G_m , and genes that were not included in G_m before permutation are added to G_m

Table 5 Correlation coefficients between *P*-values of miRNA-targeted-gene promoter hypomethylation and miRNA regulation of target genes.

Statistical test	ρ^{Pearson}	$\rho_{\log}^{\text{Pearson}}$	ρ^{Spearman}
Cell senescence	$P_{m.\text{methy}} = P_m^> \text{ vs. } P_{m.\text{regul}} = P_m^>$		
IMR90			
<i>t</i> test	0.33	0.40	0.41
<i>P</i> -values	*	*	*
Wilcoxon rank sum test	0.28	0.35	0.33
<i>P</i> -values	*	*	*
Kolmogorov-Smirnov test	0.31	0.72	0.61
<i>P</i> -values	*	*	*
MRC5			
<i>t</i> test	0.22	0.23	0.25
<i>P</i> -values	*	*	*
Wilcoxon rank sum test	0.53	0.76	0.73
<i>P</i> -values	*	*	*
Kolmogorov-Smirnov test	0.38	0.82	0.67
<i>P</i> -values	*	*	*
Differentiation	$P_{m.\text{methy}} = P_m^> \text{ vs. } P_{m.\text{regul}} = 1 - P_m^<$		
BG02			
<i>t</i> test	-0.25	-0.68	-0.67
<i>P</i> -values	*	*	*
Wilcoxon rank sum test	-0.13	-0.52	-0.53
<i>P</i> -values	2.4×10^{-9}	*	*
Kolmogorov-Smirnov test	-0.16	-0.70	-0.69
<i>P</i> -values	2.8×10^{-12}	*	*
BG03			
<i>t</i> test	-0.31	-0.57	-0.53
<i>P</i> -values	*	*	*
Wilcoxon rank sum test	-0.14	-0.23	-0.23
<i>P</i> -values	8.9×10^{-11}	*	*
Kolmogorov-Smirnov test	-0.14	-0.21	-0.19
<i>P</i> -values	1.3×10^{-10}	*	*

Various correlation coefficients between *P*-values of promoter methylation and regulation of target genes in IMR90 and MRC5 during cell senescence and in BG02 and BG03 during differentiation; *P*-values associated with the correlation coefficients are indicated. * indicates $< 2.2 \times 10^{-16}$. $P_m^>$ s were employed for cell senescence while $P_m^>$ s of promoter methylation and $1 - P_m^<$ of regulation of target genes were employed for differentiation as shown, as these combinations exhibited the most significant correlations. Genes with miRNA-targeted-gene promoter hypomethylation were recognized to be downregulated during cell senescence based on positive correlations and upregulated during differentiation based on negative correlations (for details, see Methods).

after permutation. Since both miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation are computed based on the comparison between G_m and

Table 6 Evaluation of the correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes.

Statistical test	ρ^{Pearson}	$\rho_{\log}^{\text{Pearson}}$	ρ^{Spearman}
GSM868008	$P_{m.\text{methy}} = P_m^> \text{ vs. } P_{m.\text{regul}} = P_m^>$		
<i>t</i> test	0.28	0.37	0.37
<i>P</i> -values	*	*	*
Wilcoxon rank sum test	0.18	0.32	0.28
<i>P</i> -values	4.4×10^{-16}	*	*
Kolmogorov-Smirnov test	0.16	0.70	0.54
<i>P</i> -values	1.9×10^{-12}	*	*
GSM739940	$P_{m.\text{methy}} = P_m^> \text{ vs. } P_{m.\text{regul}} = P_m^>$		
<i>t</i> test	0.11	0.40	0.34
<i>P</i> -values	3.0×10^{-6}	*	*
Wilcoxon rank sum test	0.07	0.33	0.27
<i>P</i> -values	2.5×10^{-3}	*	*
Kolmogorov-Smirnov test	0.07	0.79	0.51
<i>P</i> -values	2.7×10^{-3}	*	*
GSM375442	$P_{m.\text{methy}} = P_m^> \text{ vs. } P_{m.\text{regul}} = P_m^>$		
<i>t</i> test	0.19	0.17	0.26
<i>P</i> -values	3.0×10^{-6}	1.5×10^{-14}	*
Wilcoxon rank sum test	0.19	0.27	0.24
<i>P</i> -values	2.2×10^{-16}	*	*
Kolmogorov-Smirnov test	0.28	0.57	0.50
<i>P</i> -values	*	*	*

Correlation coefficients between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes during cell senescence shown in Table 5 were evaluated by a comparison with the results produced by different array designs (GSM868008), distinct research group, alternative measures of promoter methylation (GSM739940), and NGS (GSM375442). Independent of these factors, miRNA-targeted-gene promoter methylation was found to always be positively correlated to miRNA regulation of target genes (i.e., genes with miRNA-targeted-gene promoter hypomethylation were downregulated during cell senescence).

G_m' , miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation must be altered by the permutation.

In spite of the above discussion, one may still argue that a correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation may not be altered or may be altered very slightly by the permutation because the modification of G_m is necessary for the computation of both miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation, even though

miRNA-targeted-gene promoter methylation itself is not likely to be significant because of permutation, as demonstrated in the previous section. (i.e., see [Table 4](#)).

In order to refuse this idea, I calculated the correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation after random permutation ([Table 7](#)) and determined that random permutation drastically reduces the correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation, although it does not alter the correlation between gene expression and promoter methylation, as discussed above. This suggests that the correlation between gene expression and promoter methylation can hardly be a cause of correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation, because correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation can take either smaller or larger values while the correlation between gene expression and promoter methylation is unaltered.

All of the above findings and discussions, together with the findings and discussions in the previous section, strongly suggest that the significant correlation between miRNA-mediated regulation of target genes and miRNA-targeted-gene promoter methylation is neither an artifact generated by the complicated algorithm employed in MiRaGE, nor a bi-product of a direct correlation between gene expression and promoter methylation, but is itself a genuine biologically significant phenomenon.

Significant reciprocal relationship between miRNA expression and target gene expression

In addition to these analyses, I also confirmed a significant reciprocal relationship between miRNA expression and target

gene expression. Because it is usually believed that miRNAs downregulate target gene expression, downregulated genes should be targeted by upregulated miRNAs and vice versa. In fact, the reciprocal relationship during the senescence process of IMR90 cells has been reported^{15,16}. In this study, I observed a similar reciprocal relationship during the differentiation of BG02 and BG03 cells²⁰ ([Table 8](#)).

Thus, I conclude that miRNA-targeted-gene promoter methylation is not an artifact, but a genuine biological process. One may doubt this conclusion given the finding that miRNA-targeted-gene promoter methylation is cell-line-independent and miRNAs are believed to regulate target gene expression in a cell-line-dependent manner. Although this implies an apparent discrepancy, the correlation coefficients obtained, which were at most 0.7 to 0.8, indicate that miRNA-targeted-gene promoter methylation reflects, at most, 50% to 60% of miRNA regulation of target genes. Thus, the cell line specific miRNA regulation of target genes can act within the remaining 50% to 40%. In actuality, as can be seen in [Table 5](#), the correlations between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes have opposite signs dependent on the biological processes being considered. Therefore, the fact that cell-line-independent and miRNA-targeted-gene promoter methylation partially governs cell line dependent miRNA regulation of target genes is not a discrepancy. At the moment, I have no hypotheses that explain why miRNA-mediated regulation of target genes is the opposite between cell senescence and cell differentiation in spite of the fact that miRNA-targeted-gene promoters are similarly hypomethylated. However, as discussed above, the miRNA-mediated regulation of target genes is often reversed because of the existence of unobserved third players. The underlying biological processes, which are unknown at present, will be elucidated in the future.

Table 7 The correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes after random gene permutation.

	miRNA regulation vs miRNA-targeted-gene promoter methylation				Gene expression vs promoter methylation (P-value)
	Before random permutation (P-value)	After random permutation			
		Minimum	Maximum	(Mean P-value)	
IMR90	0.39 (2.5 × 10 ⁻¹³)	−0.127	0.133	(0.03)	−0.016 (2.27 × 10 ⁻⁵)
MRC5	0.22 (1.7 × 10 ⁻⁷)	−0.105	0.128	(0.07)	−0.003 (0.77)
BG02	−0.27 (2.0 × 10 ⁻⁷)	−0.141	0.078	(0.01)	−0.048 (9.03 × 10 ⁻⁸)
BG03	−0.30 (2.3 × 10 ⁻¹⁴)	−0.086	0.148	(0.02)	0.021 (0.02)

ρ^{Pearson} with $P_{\text{methy}} = P_m^*$ and $P_{\text{regul}} = P_m^*$ where P-values were computed by *t* test. Before random permutation: correlations between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes after random permutation and associated P-values computed from 100 random permutations ensemble assuming the normal distribution. After random permutation: minimum and maximum correlations among 100 random permutation ensembles. P-values are computed via geometric mean over 100 random permutation ensembles. Gene expression vs promoter methylation: correlation between gene expression and promoter methylation. Please note that genes are permuted such that correlation between gene expression and methylation is conserved. P-values are computed by *t* test.

Table 8 The reciprocal relationship between target gene regulation by miRNA and miRNA expression.

Cell lines	Δx_c	Correlation coefficients	<i>P</i> -value
BG02	0.00	0.18	1.9×10^{-4}
BG03	0.04	0.51	0.04

The reciprocal relationship between target gene regulation by miRNA and miRNA expression was evaluated in BG02 and BG03 cell lines during differentiation. Given that only a limited number of miRNAs are differentially expressed during differentiation, miRNAs were screened as described in the Methods section before computing a correlation. Then the correlation coefficients between differential expression of miRNA and logarithmic value of *P*-values of miRNA regulation of target genes were computed. A positive correlation implied the reciprocal relationship, that is, if the target genes are expressive, then, the miRNA itself should be suppressive (for details, see Methods).

One should understand that my objective is not to insist that miRNA up/downregulation is associated with hyper/hypomethylation of the promoter. Nor can I confirm whether miRNA mediates promoter hypermethylation or hypomethylation. To my knowledge, there are no experimental observations that illustrate how miRNA methylate target gene promoters in animal cells. However, in plants, there are some findings that mature miRNAs in nuclei can mediate DNA methylation²⁷. There are also some reports that mature miRNAs exist in, or can be transferred to the nucleus²⁸, though mediation of promoter methylation is not observed. Details in animal cells are not yet known. In the present study, I observed correlations between gene expression and promoter methylation from an miRNA-centric viewpoint. The biological mechanism mediating this correlation, however, is not clear. It is plausible that an unknown biological factor regulates both gene expression and promoter methylation, and that the correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes may simply be an outcome of indirect regulation and promoter methylation. I cannot deny this possibility, but if the correlation between miRNA-targeted-gene promoter methylation and miRNA regulation of target genes represents a real phenomena and can be found in a wide range of biological processes, there must be a biological/mechanistic underpinning of this effect.

Significant promoter hypomethylation of miR-548 miRNAs target genes

In order to better understand the biological significance of our findings regarding miRNA-targeted-gene promoter methylation, I sought to identify the specific miRNAs that target genes with hypomethylated promoters. I found that most gene promoters targeted by miR-548 miRNAs were highly hypomethylated (Table 9; here, I define miRNAs called “miR-548”

Table 9 Evaluation of miR-548 miRNAs targeting genes with miRNA-targeted-gene promoter hypomethylation.

Statistical Test	Number of miR-548 miRNAs		Mean rank
	Within top 100 ranked	Total number	
<i>t</i> test	43	68	216.2
Wilcoxon rank sum test	43	68	214.0
Kolmogorov-Smirnov test	43	68	215.0

68 miR-548 miRNAs were ranked as described in the Methods (promoter methylation was defined as the amount of methylation). Independent of the statistical methods used for computation of P_m , 43 out of 68 miR-548 miRNAs were ranked within the top-ranked 100 miRNAs. The mean rank of all miR-548 miRNAs is ~200 independent of the statistical method used. Given that the total number of miRNAs considered was 1921, these values are highly significant, $P = 2 \times 10^{-35}$.

as miR-548 miRNAs). At present, little is known about miR-548 miRNAs, which likely results from the fact that miR-548 miRNAs are primate-specific miRNAs (ps-miRNAs). Because of this, ps-miRNAs were not expected to play critical roles in fundamental biological processes like cell differentiation and development, and it has so far been widely assumed that such basic biological processes are conserved among mammals. This assumption has resulted in the exclusion of ps-miRNAs from consideration as candidates for basic and important biological processes. Thus, it is thought that the roles of these ps-miRNAs may be limited to primate-specific biological processes. Another reason that miR-548 miRNAs have not been extensively investigated is that they are generally expressed at low levels; miRNAs that play critical roles are thought to be highly expressed. For example, although miRBase stores the number of short reads detected by next generation sequencing (NGS) for various miRNAs, miR-548 miRNAs have at most 100 short reads; this is in comparison to more abundant miRNAs like those from the let-7 family, which are typically represented by several million reads. This makes it difficult to detect miR-548 miRNAs using microarray technology or sequencing. Actually, although there have been a few reports noting that miR-548 family members, including miR-548 miRNAs, play critical roles, these data come exclusively from quantitative PCR (qPCR) experiments^{29,30}.

In this study, I found that promoters of genes targeted by miR-548 miRNAs were hypomethylated. Because it is generally assumed that genes with hypomethylated promoters are expressed, the fact that promoters of target genes of miR-548 miRNAs are hypomethylated may mean that, compared to genes that are targets of other miRNAs, target genes of miR-548 miRNAs are more sensitive to changes in miRNA expression. This could explain why promoters of target genes of non-expressed miR-548 miRNAs are specifically hypomethylated.

Table 10 Ranking of miRNA regulation of target genes.

Cell lines	Target genes	Number of miR-548 miRNAs		Mean rank	P-values
		Within top 100 ranked	Total number		
IMR90	Downregulated	24	68	438.2	9×10^{-14}
MRC5	Downregulated	16	68	382.6	5×10^{-7}
BG02	Upregulated	24	68	427.8	9×10^{-14}
BG03	Upregulated	3	68	762.4	7×10^{-1}

miRNAs were ranked as described in the Methods. Target genes of miR-548 miRNAs were significantly downregulated during cell senescence (IMR90 and MRC5 cell lines) and upregulated during differentiation (BG02). Insignificant upregulation of target genes of miR-548 miRNAs in the BG03 cell line was possibly because of the weak correlation between miRNA expression and miRNA regulation of target genes (Table 8). Given that the total number of miRNAs considered was 1921, the values for IMR90, MRC5, and BG02 cell lines are highly significant.

miR-548 miRNAs represent a large set of miRNAs, with 68 family members; thus, although these miRNAs exhibit low levels of expression, the fact that there are many family members may imply that they have important biological roles. Indeed, it has been reported that potential target genes of miR-548 miRNAs play critical roles in various biological processes^{31,32}. Since it is also suggested that they originate from transposable elements (TEs), miR-548 miRNAs exhibit high sequence similarities while maintaining significant sequence diversity³¹.

During senescence processes of IMR90 and MRC5 cell lines, the target genes of miR-548 miRNAs were significantly downregulated (Table 10), whereas during differentiation of the BG02 cell line, the target genes of miR-548 miRNAs were significantly upregulated (Table 10). The fact that I observed significant changes in expression of target genes without significant changes in expression of miRNAs that target these genes implicates a role of miRNA-targeted-gene promoter hypomethylation.

Of course, the possibility remains that there are unknown biological factors that both mediate promoter methylation of miR-548 target genes and regulate gene expression targeted by miR-548 miRNAs, and all findings here are just outcomes of this hidden biological processes. Although I cannot deny this possibility, even if there are some hidden biological processes, the fact is that miRNA-548 target genes are both methylated in promoters and their regulation is robust. Future work investigating the potential underlying mechanisms are warranted.

Conclusions

Figure 1 schematically summarises the principal findings of this study. To the best of my knowledge, this is the first time that miRNA-targeted-gene promoter methylation in many types of cell lines have been observed (Tables 1 and 2). miRNA-targeted-gene hypomethylation was correlated (Tables 5 and 6) with regulation of miRNA target genes, which had a reciprocal relationship with miRNA expression; genes with miRNA-targeted-gene hypomethylated promoters were downregulated during cell senescence^{15,16} and upregulated during differentiation (Table 8). I also found that genes with miRNA-targeted-gene hypomethylated promoters were specific targets of miR-548 miRNAs (Tables 9 and 10). Regulation of target genes by miR-548 miRNAs, which were typically expressed at low levels are likely the result of miRNA-targeted-gene promoter hypomethylation. However, all findings here were correlative and thus lacked the consideration of biological background mechanism that causes the observed correlation.

List of abbreviations

TSS: transcription start sites

GEO: gene expression omnibus

bp: basepair

miRNA: microRNA

ps-miRNA: primate-specific miRNAs

TE: transposable elements

NGS: next generation sequencing

RISC: RNA-induced silencing complex

Figure 1 Schematic representing the principal findings of the present study together, as well as those reported previously. Promoters are methylated dependent upon miRNAs that target each gene (Tables 1 and 2). Genes with miRNA-targeted-gene hypomethylated promoters are downregulated during cell senescence and upregulated during cellular differentiation (Tables 5 and 6), whereas miRNAs that target genes with hypomethylated promoters were upregulated during cell senescence^{15,16} and downregulated during differentiation (Table 8). miR-548 miRNAs, which are expressed at low levels, were suggested to regulate target genes with the assistance of miRNA-targeted-gene promoter hypomethylation (Tables 9 and 10).

Competing interests

No relevant competing interests disclosed.

Author's contributions

Conceived and designed the experiments: YHT. Analyzed the data: YHT. Wrote the paper: YHT.

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Additional Files

Additional file 1 — Full list of *P*-values from the analysis of miRNA-targeted-gene promoter hypomethylation: *t* test

t test.xlsx: List of *P*-values from analysis of promoter hypomethylation of miRNA target genes calculated using the *t* test based on the amount of methylation.

Additional File 1: Supplementary data t test

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.106265>

Additional file 4 — Full list of *P*-values from the analysis of miRNA-targeted-gene promoter hypomethylation: *t* test

beta t test.xlsx: List of *P*-values from the analysis of promoter hypomethylation of miRNA target genes calculated using the *t* test based on β -values.

Additional File 4: Supplementary data beta t test

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.106268>

Additional file 2 — Full list of *P*-values from the analysis of miRNA-targeted-gene promoter hypomethylation: Wilcoxon rank sum test

Wilcoxon rank sum test.xlsx: List of *P*-values from the analysis of promoter hypomethylation of miRNA target genes calculated using the Wilcoxon rank sum test based on the amount of methylation.

Additional File 2: Supplementary data Wilcoxon rank sum test

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.106266>

Additional file 5 — Full list of *P*-values from the analysis of miRNA-targeted-gene promoter hypomethylation: Wilcoxon rank sum test

beta Wilcoxon rank sum test.xlsx: List of *P*-values from the analysis of promoter hypomethylation of miRNA target genes calculated using the Wilcoxon rank sum test based on β -values.

Additional File 5: Supplementary data beta Wilcoxon rank test

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.106269>

Additional file 3 — Full list of *P*-values from the analysis of miRNA-targeted-gene promoter hypomethylation: Kolmogorov-Smirnov test

Kolmogorov Smirnov test.xlsx: List of *P*-values from the analysis of promoter hypomethylation of miRNA target genes calculated using the Kolmogorov-Smirnov test based on the amount of methylation.

Additional File 3: Supplementary data Kolmogorov Smirnov test

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.106267>

Additional file 6 — Full list of *P*-values from the analysis of miRNA-targeted-gene promoter hypomethylation: Kolmogorov-Smirnov test

beta Kolmogorov Smirnov test.xlsx: List of *P*-values from the analysis of promoter hypomethylation of miRNA target genes calculated using the Kolmogorov-Smirnov test based on β -values.

Additional File 6: Supplementary data beta Kolmogorov Smirnov test

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.106270>

References

- Golbabapour S, Abdulla MA, Hajrezaei M: A concise review on epigenetic regulation: insight into molecular mechanisms. *Int J Mol Sci.* 2011; **12** (12): 8661–8694.
- Suzuki MM, Bird A: DNA methylation landscapes: provocative insights from epigenomics. *Nat Rev Genet.* 2008; **9** (6): 465–476.
- Bartova E, Krejci J, Harnicarova A, *et al*: Histone modifications and nuclear architecture: a review. *J Histochem Cytochem.* 2008; **56** (8): 711–721.
- Pabo CO, Sauer RT: Transcription factors: structural families and principles of DNA recognition. *Annu Rev Biochem.* 1992; **61**: 1053–1095.
- He L, Hannon GJ: MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet.* 2004; **5** (7): 522–531.
- Das PM, Singal R: DNA methylation and cancer. *J Clin Oncol.* 2004; **22** (22): 4632–4642.
- Boks MP, Derks EM, Weisenberger DJ, *et al*: The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS One.* 2009; **4** (8): e6767.
- Berardi E, Pues M, Thorrez L, *et al*: microRNAs in ES Cell Differentiation. *Am J Physiol Heart Circ Physiol.* 2012; **303** (8): H931–9.
- Etheridge A, Lee I, Hood L, *et al*: Extracellular microRNA: A new source of biomarkers. *Mutat Res.* 2011; **717** (1–2): 85–90.
- Lopez-Serra P, Esteller M: DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer. *Oncogene.* 2012; **31** (13): 1609–1622.
- Su Z, Xia J, Zhao Z: Functional complementation between transcriptional methylation regulation and post-transcriptional microRNA regulation in the human genome. *BMC Genomics.* 2011; **12** Suppl 5: S15.
- Sinha S, Adler AS, Field Y, *et al*: Systematic functional characterization of cis-regulatory motifs in human core promoters. *Genome Res.* 2008; **18** (3): 477–488.
- Saito T, Sætrum P: Target gene expression levels and competition between transfected and endogenous microRNAs are strong confounding factors in microRNA high-throughput experiments. *Silence.* 2012; **3**: 3.
- Nazor KL, Altun G, Lynch C, *et al*: Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. *Cell Stem Cell.* 2012; **10** (5): 620–634.
- Taguchi YH: Inference of Target Gene Regulation via miRNAs during Cell Senescence by Using the MiRaGE Server. *Aging Dis.* 2012; **3** (4): 301–6.
- Taguchi YH: Inference of target gene regulation via miRNAs during cell senescence by using the MiRaGE Server. In *Emerging Intelligent Computing Technology and Applications*. Edited by Huang DS, Gupta P, Zhang X, Premaratne P, Springer: Heidelberg 304: 2012; 441–446.
- Ohm JE, Mali P, Van Neste L, *et al*: Cancer-related epigenome changes associated with reprogramming to induced pluripotent stem cells. *Cancer Res.* 2010; **70** (19): 7662–7673.
- Deng J, Shoemaker R, Xie B, *et al*: Targeted bisulfite sequencing reveals changes in DNA methylation associated with nuclear reprogramming. *Nat Biotechnol.* 2009; **27** (4): 353–360.
- Krueger F, Andrews SR: Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics.* 2011; **27** (11): 1571–1572.
- Stadler B, Ivanovska I, Mehta K, *et al*: Characterization of microRNAs involved in embryonic stem cell states. *Stem Cells Dev.* 2010; **19** (7): 935–950.
- Yoshizawa M, Taguchi YH, Yasuda J, *et al*: Inference of Gene Regulation via miRNAs During ES Cell Differentiation Using MiRaGE Method. *Int J Mol Sci.* 2011; **12**: 9265–9276.
- R Core Team: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 2012.
- Benjamini Y, Hochberg Y: Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc B.* 1995; **57**: 289–300.
- Lu TP, Lee CY, Tsai MH, *et al*: miRSystem: An Integrated System for Characterizing Enriched Functions and Pathways of MicroRNA Targets. *PLoS One.* 2012; **7** (8): e42390.
- Shahab SW, Matyunina LV, Hill CG, *et al*: The effects of MicroRNA transfections on global patterns of gene expression in ovarian cancer cells are functionally coordinated. *BMC Med Genomics.* 2012; **5**: 33.
- Ebert MS, Neilson JR, Sharp PA: MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods.* 2007; **4** (9): 721–726.
- Khraiweh B, Arif MA, Seumel GI, *et al*: Transcriptional control of gene expression by microRNAs. *Cell.* 2010; **140**: 111–122.
- Nishi K, Nishi A, Nagasawa T, *et al*: Human TNRC6A is an Argonaute-navigator protein for microRNA-mediated gene silencing in the nucleus. *RNA* 2013; **19**: 17–35.
- Marasa BS, Srikantan S, Martindale JL, *et al*: MicroRNA profiling in human diploid fibroblasts uncovers miR-519 role in replicative senescence. *Aging (Albany NY).* 2010; **2** (6): 333–343.
- Yang C, Wang C, Chen X, *et al*: Identification of seven serum microRNAs from a genome-wide serum microRNA expression profile as potential noninvasive biomarkers for malignant astrocytomas. *Int J Cancer.* 2013; **132** (1): 116–27.
- Lin S, Cheung WK, Chen S, *et al*: Computational identification and characterization of primate-specific microRNAs in human genome. *Comput Biol Chem.* 2010; **34** (4): 232–241.
- Liang T, Guo L, Liu C, *et al*: Genome-wide analysis of mir-548 gene family reveals evolutionary and functional implications. *J Biomed Biotechnol.* 2012; **2012**: 679563.

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Pål Sætrum

Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway

Although this article's topic is interesting, I found that its contents were confusing and inaccessible. I also have some reservations regarding the methods.

- **The abstract is misleading.**

The author states in the abstract: *"It was found that promoter methylation was affected by miRNA targeting."* I find this statement very misleading, as the author has not shown that miRNA targeting has an effect on promoter methylation. What the authors' results do suggest is that the promoters of genes that are predicted to be miRNA targets tend to be hypomethylated compared to promoters of genes predicted not to be miRNA targets. Although this correlation is an interesting observation in itself (also previously made by [Su et al., 2011](#)), the author provides no compelling evidence for miRNA targeting and regulation being the cause of (or even influence) the genes' reduced promoter methylation. Specifically, the author neither demonstrates that loss (or increase) of miRNA regulation in general nor that loss (or increase) of regulation by specific miRNAs affects promoter methylation status. Instead, the author's results indicate that predicted miRNA regulation apparently can have both a positive and negative correlation with promoter methylation status (Table 5 and Table 7). Consequently, the author's current results only support that predicted miRNA targets tend to have hypomethylated promoters in the specific cell lines used in the author's study.

- **There are no figures to illustrate analyses, show data trends, and visualize correlations.**

The author consistently presents his results as correlation coefficients and p-values. These values are important, but also hide important aspects of the data, such as the magnitude of a significant difference and the trend of or the number of observations contributing to a significant correlation. To make his results more accessible, the author should at least create a figure that compares the distributions of promoter methylation for miRNA target and non-target genes (Table 3). Moreover, some of the author's analyses, such as the correlations of p-values in Tables 5-6, are fairly complex. A figure that shows the underlying data and illustrates the different steps in the analyses would make the results more accessible.

- **The analyses apparently ignore miRNA expression.**

Presumably, a miRNA has to be expressed to have a role in gene regulation – whether that role is post-transcriptional or otherwise. It is unclear, however, whether the author takes miRNA expression into account in his analyses. For example, in Table 1, the author has apparently included all 1921 mature miRNAs from miRBase 18.0 in his analyses, but I doubt that all of these are expressed in all the 271 cell lines. One reason for including all miRNAs may be that the author has no data on which miRNAs are expressed in the different cell lines. Nevertheless, as many of the miRNAs will likely not be expressed in a given cell line, a large fraction of the 520,591 combinations tested appear irrelevant. Similarly, for the analyses presented in Table 5, where the author does have data on the miRNAs that are expressed, the author provides no information on the miRNAs actually used in the analysis. Finally, in the last result section, the author specifically studies the miR-548 family, which has very low expression levels in the cells where it has been detected. The author seems to suggest that the predicted targets of the miR-548 family are especially likely to be hypo-methylated and concludes that *“Regulation of target genes by miR-548 miRNAs, which were typically expressed at low levels are likely the result of miRNA-targeted-gene promoter hypomethylation.”* The section also contains several confusing statements such as *“Because [...] genes with hypomethylated promoters are expressed, the fact that promoters of target genes of miR-548 miRNAs are hypomethylated may mean that, compared to genes that are targets of other miRNAs, target genes of miR-548 miRNAs are more sensitive to changes in miRNA expression. This could explain why promoters of target genes of non-expressed miR-548 miRNAs are specifically hypomethylated.”* If, for arguments sake, miRNAs really affect promoter methylation, I still fail to understand why a lowly expressed miRNA such as miR-548 should have a stronger effect than other more highly expressed miRNAs, as the author seem to suggest. Instead, I believe that the miR-548 family appear to be significantly associated with hypomethylated promoters compared with other miRNAs because of a bias in the author’s analysis. Specifically, many of the members of the miR-548 family have identical seed sequences and will therefore be predicted to target the same genes (the number of unique 7mer seeds in the miR-548 family in miRBase 18.0 is 33), but the author does not seem to correct for this identical targeting in his ranking analyses (Table 9 and 10). Overall, the author should take into account both miRNA expression and similar or identical miRNA targeting in his analyses.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 09 Sep 2013

Y-h. Taguchi, Chuo University, Tokyo, Japan

Thank you very much for your efforts to review my article. In the following, I would like to reply to your valuable comments point by point.

- Although this article’s topic is interesting, I found that its contents were confusing and inaccessible. I also have some reservations regarding the methods. The abstract is misleading. The author states in the abstract: “It was found that promoter methylation was affected by miRNA

targeting.” I find this statement very misleading, as the author has not shown that miRNA targeting has an effect on promoter methylation.

I have modified the abstract to emphasize that what I found was not causalities but solely correlations.

- What the authors’ results do suggest is that the promoters of genes that are predicted to be miRNA targets tend to be hypomethylated compared to promoters of genes predicted not to be miRNA targets. Although this correlation is an interesting observation in itself (also previously made by Su et al., 2011), the author provides no compelling evidence for miRNA targeting and regulation being the cause of (or even influence) the genes’ reduced promoter methylation. Specifically, the author neither demonstrates that loss (or increase) of miRNA regulation in general nor that loss (or increase) of regulation by specific miRNAs affects promoter methylation status. Instead, the author’s results indicate that predicted miRNA regulation apparently can have both a positive and negative correlation with promoter methylation status (Table 5 and Table 7). Consequently, the author’s current results only support that predicted miRNA targets tend to have hypomethylated promoters in the specific cell lines used in the author’s study.

I have recently published a paper that demonstrates miRNA-targeted-promoter methylation can be found also in human brain [PMID: 23725297]. Thus, I believe that miRNA-targeted-promoter methylation is observed not only in the specific cell lines, but also in other biological situations.

- There are no figures to illustrate analyses, show data trends, and visualize correlations.

I have added additional file 8 that shows the scatter plots corresponding to Tables 5 and 6.

- The author consistently presents his results as correlation coefficients and p-values. These values are important, but also hide important aspects of the data, such as the magnitude of a significant difference and the trend of or the number of observations contributing to a significant correlation. To make his results more accessible, the author should at least create a figure that compares the distributions of promoter methylation for miRNA target and non-target genes (Table 3).

I have added additional file 8 in order to fulfill the reviewer’s requirements.

- Moreover, some of the author’s analyses, such as the correlations of p-values in Tables 5-6, are fairly complex. A figure that shows the underlying data and illustrates the different steps in the analyses would make the results more accessible.

I have added Fig. 1 in order to fulfill the reviewer’s requirements.

- The analyses apparently ignore miRNA expression. Presumably, a miRNA has to be expressed to have a role in gene regulation – whether that role is post-transcriptional or otherwise. It is unclear, however, whether the author takes miRNA expression into account in his analyses. For example, in Table 1, the author has apparently included all 1921 mature miRNAs from miRBase 18.0 in his analyses, but I doubt that all of these are expressed in all the 271 cell lines. One reason

for including all miRNAs may be that the author has no data on which miRNAs are expressed in the different cell lines. Nevertheless, as many of the miRNAs will likely not be expressed in a given cell line, a large fraction of the 520,591 combinations tested appear irrelevant. Similarly, for the analyses presented in Table 5, where the author does have data on the miRNAs that are expressed, the author provides no information on the miRNAs actually used in the analysis.

In Tables 1 and 5, I have considered all miRNAs in miRBase whether they are expressed or not. I think that the amount of miRNA expression is not always a primary factor with regards to the control of miRNA regulation for the target genes. References 36 and 37 in the article support this point. In addition to this, instead of the miRNA expression, I demonstrated that miRNA-targeted-gene promoter methylation is related to seed region features (see Fig. 2, additional file 9, and section “miRNA-targeted-genes promoter methylation is correlated to miRNA seed region features”). Although I do not intend to insist that miRNA seed region features can control miRNA-targeted-gene promoter methylation, I think that the amount of miRNA expression does not always have to be the only factor which controls the miRNA-regulation of target genes.

- Finally, in the last result section, the author specifically studies the miR-548 family, which has very low expression levels in the cells where it has been detected. The author seems to suggest that the predicted targets of the miR-548 family are especially likely to be hypo-methylated and concludes that “Regulation of target genes by miR-548 miRNAs, which were typically expressed at low levels are likely the result of miRNA-targeted-gene promoter hypomethylation.” The section also contains several confusing statements such as “Because [...] genes with hypomethylated promoters are expressed, the fact that promoters of target genes of miR-548 miRNAs are hypomethylated may mean that, compared to genes that are targets of other miRNAs, target genes of miR-548 miRNAs are more sensitive to changes in miRNA expression. This could explain why promoters of target genes of non-expressed miR-548 miRNAs are specifically hypomethylated.” If, for arguments sake, miRNAs really affect promoter methylation, I still fail to understand why a lowly expressed miRNA such as miR-548 should have a stronger effect than other more highly expressed miRNAs, as the author seem to suggest. Instead, I believe that the miR-548 family appear to be significantly associated with hypomethylated promoters compared with other miRNAs because of a bias in the author’s analysis.

As mentioned above, I do not think that the amount of miRNA expression is always the only factor which controls miRNA-regulation of target genes. Apparently, miR-548 target genes have more distinct seed region features than other miRNAs. I think that this may be the reason why low amounts of miR-548 expression can be related to miRNA-targeted-gene promoter methylation (see Fig. 3, Table 11, and section “miRNA-targeted-genes promoter methylation is correlated to miRNA seed region features”).

- Specifically, many of the members of the miR-548 family have identical seed sequences and will therefore be predicted to target the same genes (the number of unique 7mer seeds in the miR-548 family in miRBase 18.0 is 33), but the author does not seem to correct for this identical targeting in his ranking analyses (Table 9 and 10). Overall, the author should take into account both miRNA expression and similar or identical miRNA targeting in his analyses.

I do not think that merging seed-sharing-miRNAs into one is always the only correct way to

predict miRNA regulation of target genes. Please see the newly added section "Treatment of shared miRNA seed sequences" and Appendix. In addition to this, my MiRaGE method - which does not merge seed-sharing-miRNAs into one - was applied to many biological processes, and I found that it works reasonably well (see References 15, 16, and 21 from the article).

I would like to thank the reviewer, whose comments have greatly improved the quality of this paper. I would be glad if the present version is approved by the reviewer.

Competing Interests: No competing interests were disclosed.

Reviewer Report 14 May 2013

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Denis Dupuy

Institut Europeen de Chimie et Biologie, Pessac, France

I had initially understood that the title of this study could be: "*Correlation between promoter methylation and miRNA regulation of target genes.*" Which in simpler terms could mean that miRNA targeted genes tend to have more hypomethylated promoters (or hypermethylated maybe) than expected by chance.

In the current form it is still unclear to me how the data actually support this conclusion.

The lack of illustration makes the article hard to follow, the author present the results of statistical tests without providing a clear description of what effect he is actually evaluating. And on further reading it seems that the objective of this study is largely incomprehensible to me.

The first question comes in the dataset compilation: how was it done and what were the criteria used to accept or reject data used in the study?

A figure representing the process of dataset generation would be welcome.

The author states: "***I tested profiles from this dataset to confirm that obtained results were not research group dependent.***" How was this done? It is well known that biological experiments on a genome scale are inherently noisy so results even within the same group will not be the exact replicate of one another. What was considered acceptable to create a dataset compendium? Was any normalization applied? Was there an a posteriori check that the independent sets were behaving in the same way as the compendium? If not, would the same conclusion have been obtained by analyzing them separately? Is only part of the data driving the conclusion and can it

be explained by the different cell lines?

Elsewhere, the author states ***"It should be noted that genes that were not the targets of any miRNAs were totally excluded from the analysis"*** which makes me think that I totally missed the initial point of this work as I thought the objective was to test the hypothesis that promoter methylation status was related to miRNA targeting of the produced messenger. In this, genes that are not targets of miRNA would be useful as a control group.

Even if the author is trying to demonstrate a relationship between the amount of miRNA regulation and methylation, non-target genes would be an essential control.

The second question arises from the choice of the values used for methylation. The author states: *"I employed a method in which total methylation values were used(..) because I found that P-values computed when using methylation data were more strongly correlated to the P-values calculated from target gene miRNA regulation data, which is likely due to the fact that the frequency of CpGs is also related to miRNA targeting"*

and later:

" β i was the ratio of methylated sites to unmethylated sites, (...) were deposited in the public datasets used, their use could not be avoided; however, as a result, the correlation with target gene miRNA regulation was substantially decreased. An explanation for this is noted above."

"Obtaining a better P-value", is not an acceptable rationale for changing the way the methylation of a promoter is "typically represented".

The reason why the association between CpG island associated genes and miRNA targeting explains the difference in P-values between using total methylation values and proportion of methylated sites, is not obvious. It hints at a different behavior between CpG and non CpG promoters, would it not then make sense to process them separately?

It seems that the two ways of representing the data mean very different things and that decision could greatly affect the conclusions that can be extracted from the results. If I understand correctly, with the absolute number method a promoter with 2/3 methylated sites would be counted the same as a 2/10 yet in one case one could consider the first to be 66% methylated and the other 20%. The implication of this choice on the results of the study should be discussed more thoroughly.

In table 1, if the top part is indeed calculated with ratio and bottom with absolute, what were the thresholds to be counted as hypomethylated in each case?

What does the "number of miRNA" actually represent? The author indicates that he multiplied the number of miRNA (1971) by the number of cell lines included (271). What does this product represent in the end? Why were the datapoints not aggregated by promoter and/or miRNA in the context of this study?

Table 3 for example is symptomatic of how the lack of illustration makes the article hard to follow: ***"P-values that represent the difference of promoter methylation between miRNA target genes"***

and miRNA non-target genes.” The P-value doesn't represent a “difference in promoter methylation”, it evaluates the likelihood of the observation happening by chance given the dataset distribution. But what is the observation here? Are promoters of genes targeted by miRNA 60% methylated vs 58% for the non targeted? (is it 10 vs 87?)

Are we considering a very slight effect with a very strong statistical significance or a very obvious strong effect for which the statistical test is not even needed to be convinced of the solidity of the result?

Moreover, just below that table the author explains why he doesn't discriminate between hypo and hypermethylation of promoters. That makes table 3 very puzzling; is this P-value evaluating the likelihood of miRNA targeted gene promoters to be significantly BOTH over and/or under methylated relative to non-targeted genes?

Table 4 has been added as a response to my previous comment however it seems to bring further question as to what “miRNA-targeted-gene promoter methylation” really means. My first comment is that this control should not constitute a separate table but an additional entry in the original table. Secondly, there are many ways to generate random shuffling of the data that will destroy the “signal”, however for a random shuffling to be a valid control of the biological significance of the observation, the randomization should be performed in a way that preserves at least part of the actual data structure. For example, if one works on a scale free distribution and discovers biological associations within this distribution, one would not generate a shuffled set as control that would have a gaussian distribution but instead would have to preserve the scale-free distribution. In this case, no details are provided regarding what steps have been taken to ensure that the randomization preserved any of the original data structure.

It is hard to believe what part of the data structure is conserved in this randomization when in the actual data between 72% to 92% of the >500,000 items studied are found to be significant (Table 1) and only 3 to 6 /500,000 have that characteristic in Table 4.

Similarly, in Table 5 “**Correlation coefficients between P-values(..)**” it is unclear what the numbers actually represent. I am familiar with measuring the P-value of a correlation coefficient through multiple randomly shuffled distributions to insure the significance of the observed correlation. But it is unclear to me what a correlation between P-values actually represents since they could well be associated with opposite effects.

Finally, Figure 1 that should bear the take home message doesn't provide a better idea of what the conclusions of the paper are: the top part shows two genes targeted by different numbers of miRNA having different levels of methylation. From this it seems that the correlation found is actually between the amount of regulatory miRNA and hypomethylation, but how this was derived from table 1 and 2 is totally unclear. What happens in the boxed part of the figure is even more confusing as it shows a young cell, with 3 (expressed?) genes, becoming a senescent cell, expressing only one with methylation unchanged even though the amount of mir-548 targeting is represented higher.

Meanwhile, the opposite is the case for the differentiation scheme. All the represented promoters (?) in the box seem to be hypomethylated regardless of the number of miRNA targeting the corresponding mRNA in total opposition to what the study seems to claim.

I think overall the methodology and lack of illustrations obfuscate the point for readers. Non-specialists and specialists alike will find it hard to follow the author's reasoning for want of clarity in the methods used. A scientific paper should strive to present the data in a way that can convince experts of the field and also explain to non specialists how the observed results lead to the presented conclusions. I feel that on both accounts this manuscript is not reaching these goals.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 16 May 2013

Y-h. Taguchi, Chuo University, Tokyo, Japan

Dear Denis,

Thank you very much for your efforts to review my paper. Here I respond to you point by point. If these responses resolve disagreements between us and then if you can approve my manuscript, I will be happy. Alternatively, you may suggest how to revise my manuscript such that you can approve it.

>I had initially understood that the title of this study could be: "Correlation between promoter methylation and miRNA regulation of target genes." Which in simpler terms could mean that miRNA targeted genes tend to have more hypomethylated promoters (or hypermethylated maybe) than expected by chance. >In the current form it is still unclear to me how the data actually support this conclusion.

Unfortunately, I did not intend to conclude that "miRNA targeted genes tend to have more hypomethylated promoters (or hypermethylated maybe) than expected by chance", but tried to conclude that "a set of genes targeted by a miRNA A is differently hypo/hypermethylated than a set of genes not targeted by the miRNA A but targeted by any other miRNAs". These two are very different.

>The lack of illustration makes the article hard to follow, the author present the results of statistical tests without providing a clear description of what effect he is actually evaluating. And on further reading it seems that the objective of this study is largely incomprehensible to me.

What I have evaluated is, as mentioned in the main text;

- 1.) "a set of genes targeted by a miRNA A is differently hypo/hypermethylated than a set of genes not targeted by the miRNA A but targeted by any other miRNAs"
- 2.) "the correlation of P-values between miRNA-targeting-promoter methylation (in the sense denoted in 1.) and miRNA-mediated regulation of target genes". I also defined these two using equations, more exactly. I would be glad if you can mention in which point you

are not sure.

>The first question comes in the dataset compilation: how was it done and what were the criteria used to accept or reject data used in the study? >A figure representing the process of dataset generation would be welcome.

As I have mentioned in the Methods section (mRNA and miRNA expression profiles), what I have done is to just make mean and variance, zero and one, respectively. I have never done any other treatments. Thus, I assumed that no detailed explanations were required. If I was not correct, please advise me about how to to prepare suitable figures.

>The author states: "I tested profiles from this dataset to confirm that obtained results were not research group dependent." How was this done? It is well known that biological experiments on a genome scale are inherently noisy so results even within the same group will not be the exact replicate of one another. What was considered acceptable to create a dataset compendium? Was any normalization applied? Was there an a posteriori check that the independent sets were behaving in the same way as the compendium? If not, would the same conclusion have been obtained by analyzing them separately? Is only part of the data driving the conclusion and can it be explained by the different cell lines?

I have compared one experiment with another experiment, only using the correlation coefficients of P-values. Almost all analyses were performed in P-value based. As you pointed out, raw values like gene expression and promoter methylation are hard to compare between different experiments. All success in this study is achieved by the employment of P-value based approaches.

>Elsewhere, the author states "It should be noted that genes that were not the targets of any miRNAs were totally excluded from the analysis" which makes me think that I totally missed the initial point of this work as I thought the objective was to test the hypothesis that promoter methylation status was related to miRNA targeting of the produced messenger. In this, genes that are not targets of miRNA would be useful as a control group.

>Even if the author is trying to demonstrate a relationship between the amount of miRNA regulation and methylation, non-target genes would be an essential control.

As I have mentioned above, the purpose of this study was to compare promoter methylation between genes targeted by different miRNAs. Thus, non-target genes cannot be any controls in this context. However, since you require me to clearly point this out, I show this in Table 3.

>The second question arises from the choice of the values used for methylation. The author states: "I employed a method in which total methylation values were used(..) because I found that P-values computed when using methylation data were more strongly correlated to the P-values calculated from target gene miRNA regulation data, which is likely due to the fact that the frequency of CpGs is also related to miRNA targeting"

>and later:

>" β i was the ratio of methylated sites to unmethylated sites, (...) were deposited in the public datasets used, their use could not be avoided; however, as a result, the correlation with target

gene miRNA regulation was substantially decreased. An explanation for this is noted above.”
>“Obtaining a better P-value”, is not an acceptable rationale for changing the way the methylation of a promoter is “typically represented”.

I did not insist that “Obtaining a better P-value” is the criterion to choose which definition of methylation should be employed. I wrote that better correlation of P-values between miRNA-targeting-specific promoter methylation and miRNA regulation of target genes is the criterion to choose which definition of methylation should be employed.

>The reason why the association between CpG island associated genes and miRNA targeting explains the difference in P-values between using total methylation values and proportion of methylated sites, is not obvious. It hints at a different behavior between CpG and non CpG promoters, would it not then make sense to process them separately?

As I have mentioned in the manuscript, there are some differences. However, the correlation between P-values for miRNA-targeting-specific promoter methylation and P-values for miRNA-mediated regulation of target genes is significant. In fact, for GSM739940, I had to employ β -values, but the correlation is still significant (see Table 6). Thus, I think that selection of values that represent promoter methylation to be investigated is not essential for the purpose of this manuscript. If you think that you need more evaluations, please advise me about what kind of evaluation is useful for you to be satisfied.

>It seems that the two ways of representing the data mean very different things and that decision could greatly affect the conclusions that can be extracted from the results. If I understand correctly, with the absolute number method a promoter with 2/3 methylated sites would be counted the same as a 2/10 yet in one case one could consider the first to be 66% methylated and the other 20%. The implication of this choice on the results of the study should be discussed more thoroughly.

As I have written in the above, since both ratio and amount of methylation work more or less well, I do not think that “decision could greatly affect the conclusions”. However, if you can suggest some kind of additional evaluations, I am happy to perform this so as to fulfill your requirements.

>In table 1, if the top part is indeed calculated with ratio and bottom with absolute, what were the thresholds to be counted as hypomethylated in each case?

As I have written in the text, I employed P-values to check if the promoters are hyper/hypomethylated. Threshold of P-values is, as denoted in the manuscript, $P < 0.05$ after adjusted based on BH criterion.

>What does the “number of miRNA” actually represent? The author indicates that he multiplied the number of miRNA (1971) by the number of cell lines included (271). What does this product represent in the end? Why were the datapoints not aggregated by promoter and/or miRNA in the context of this study?

As I have written in the manuscript, P-values are attributed not to each gene but to each

miRNA. Thus, in the framework of the present study, it is impossible to attribute P-values to each promoter. Also, P-values are attributed to miRNAs in each cell line, because each cell line has a different pattern of promoter methylation. Thus, in some cell lines a miRNA's target genes' promoters are significantly hyper/hypomethylated, while they may not be in other cell lines. Thus, to each miRNA, the number of P-values attributed is the number of cell line. Thus, I get the number of cell lines times number of miRNAs P-values in total.

>Table 3 for example is symptomatic of how the lack of illustration makes the article hard to follow: "P-values that represent the difference of promoter methylation between miRNA target genes and miRNA non-target genes." The P-value doesn't represent a "difference in promoter methylation", it evaluates the likelihood of the observation happening by chance given the dataset distribution. But what is the observation here? Are promoters of genes targeted by miRNA 60% methylated vs 58% for the non targeted? (is it 10 vs 87?)

It depends upon the statistical test employed, since I used three statistical tests. For the t-test, your expectation is more or less true, since the t-test compares mean values between two sets (although I used amount of methylation, not ratio, as denoted in the text) . For other tests, other statistical values are used in order to check significant difference.

>Are we considering a very slight effect with a very strong statistical significance or a very obvious strong effect for which the statistical test is not even needed to be convinced of the solidity of the result?

As I have written in the above, the essence of this study is to consider the correlation of P-values. This is the only useful variable to compare different experiments. Thus, I cannot answer your question. Using only P-values, I cannot distinguish between two cases: "a very slight effect with a very strong statistical significance" and "a very obvious strong effect for which the statistical test is not even needed to be convinced of the solidity of the result", but considering other variables make the comparison between the different experiments impossible, as you suggested in the above. However, as I have mentioned in the above, employment of P-value based analyses is essential to derive conclusions in this paper.

>Moreover, just below that table the author explains why he doesn't discriminate between hypo and hypermethylation of promoters. That makes table 3 very puzzling; is this P-value evaluating the likelihood of miRNA targeted gene promoters to be significantly BOTH over and/or under methylated relative to non-targeted genes?

Yes, I did not distinguish between these two cases. However, if you wish, it is not difficult to investigate it if I used a one-sided test.

>Table 4 has been added as a response to my previous comment however it seems to bring further question as to what "miRNA-targeted-gene promoter methylation" really means. My first comment is that this control should not constitute a separate table but an additional entry in the original table.

I am not sure what kind of information you need. Please advise me what to do.

>Secondly, there are many ways to generate random shuffling of the data that will destroy the "signal", however for a random shuffling to be a valid control of the biological significance of the observation, the randomization should be performed in a way that preserves at least part of the actual data structure. For example, if one works on a scale free distribution and discovers biological associations within this distribution, one would not generate a shuffled set as control that would have a gaussian distribution but instead would have to preserve the scale-free distribution. In this case, no details are provided regarding what steps have been taken to ensure that the randomization preserved any of the original data structure.

P-values are computed based upon the comparison of promoter methylation between genes targeted by a miRNA A and genes not targeted by the miRNA A but targeted by any other miRNAs. Permutation was performed simply between these two sets. In this occasion, what should be conserved? I did not find anything to be conserved on this occasion. I would be happy if you can suggest it.

>It is hard to believe what part of the data structure is conserved in this randomization when in the actual data between 72% to 92% of the >500,000 items studied are found to be significant (Table 1) and only 3 to 6 /500,000 have that characteristic in Table 4.

I do not think that permutation can conserve anything. I would be glad if you can suggest what to be conserved.

>Similarly, in Table 5 "Correlation coefficients between P-values(..)" it is unclear what the numbers actually represent. I am familiar with measuring the P-value of a correlation coefficient through multiple randomly shuffled distributions to insure the significance of the observed correlation. But it is unclear to me what a correlation between P-values actually represents since they could well be associated with opposite effects.

Since I used one-sided tests as denoted in the manuscript, P-values can distinguish between opposite effects.

>Finally, Figure 1 that should bear the take home message doesn't provide a better idea of what the conclusions of the paper are: the top part shows two genes targeted by different numbers of miRNA having different levels of methylation. From this it seems that the correlation found is actually between the amount of regulatory miRNA and hypomethylation, but how this was derived from table 1 and 2 is totally unclear. What happens in the boxed part of the figure is even more confusing as it shows a young cell, with 3 (expressed?) genes, becoming a senescent cell, expressing only one with methylation unchanged even though the amount of mir-548 targeting is represented higher.

>Meanwhile, the opposite is the case for the differentiation scheme. All the represented promoters (?) in the box seem to be hypomethylated regardless of the number of miRNA targeting the corresponding mRNA in total opposition to what the study seems to claim.

Figure 1 is just an illustration about what "can" happen. Since I investigated only P-values, I cannot strictly decide what has happened exactly. There are no ways to derive Figure 1 from Table 1 and 2 directly. I think that it helps readers to imagine what is the example of what happened in the cell. If you think that it is not suitable, I will remove it.

>I think overall the methodology and lack of illustrations obfuscate the point for readers. Non-specialists and specialists alike will find it hard to follow the author's reasoning for want of clarity in the methods used. A scientific paper should strive to present the data in a way that can convince experts of the field and also explain to non specialists how the observed results lead to the presented conclusions. I feel that on both accounts this manuscript is not reaching these goals.

I hope that reading my responses, you can approve my manuscript as it is or suggest ways to revise my manuscript so as to fulfill your requirements. I believe that what I have found is worthwhile publishing, thus it is better to be indexed by your approval.

Yours, Tag.

Prof. Y-h. Taguchi, Department of Physics, Chuo University, Tokyo 112-8551, Japan

Competing Interests: No competing interests were disclosed.

Version 1

Reviewer Report 06 February 2013

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I find that the often-used term of 'miRNA-targeting-specific promoter methylation' (sic) should be replaced by a less confusing expression, e.g. 'miRNA-targeted-gene promoter methylation', as 'targeting' and 'specific' are redundant and confuse the point. Specifically, the grammatical construction would seem to indicate that there are hypomethylated promoter specific targeting miRNAs (title of a subsection) which doesn't seem to be what the author means. Otherwise, the term 'specific' could also lead to believe that there is a dedicated mechanism for demethylation of these subset of (miRNA targeted) promoters that would be distinct from other methylation pathways. As there is no way to know if it is the case or whether there is a unique activity biased toward genes submitted to post transcriptional regulation.

Moreover, the use of both: 'miRNA-targeting-specific promoter hypomethylation' and 'miRNA-targeting-specific promoter methylation' adds to the confusion as these should indicate opposite trends or mechanisms.

The author states that; '[MiRaGE] was designed to infer the differential expression of miRNA target genes between two experimental conditions based on the expression profiles of the target genes in question', this is not exactly right: as stated on the MiRaGE page, 'MiRaGE method is the method used to infer gene expression regulation via miRNA based upon target gene expression.' Thus, it doesn't infer the expression of target genes! It infers the contribution of miRNA to the **measured** expression levels. So by replacing the expression levels by the methylation status of the promoter the author is trying to uncover a link between miRNA expression and the methylation of their target genes.

I feel that a much needed control would be required to see how the correlation between methylation status and miRNA target gene expression differs from the correlation between non-targeted genes with similar expression levels.

Another potentially damning issue is raised in Table 3: 'It was clear that promoter methylation and miRNA regulation of target genes were significantly correlated during both cell senescence and differentiation'. Another way to interpret this would be that the expression level of those genes is correlated with the methylation level of their promoter (which is well reported in the literature), this could be considered an alternative explanation to the modification of expression levels observed in those genes (i.e. not through miRNA regulation but through chromatin remodeling). This should warrant the use of a control set of non-miRNA targeted genes to check if the promoter methylation is truly 'specific' of miRNA targeting.

More troubling is this statement: 'correlation coefficients exhibited opposite directionalities in cell senescence and differentiation. This means that genes with miRNA-targeting-specific promoter hypomethylation are downregulated during cell senescence, but upregulated during differentiation'. It seems that there should be a more in depth discrimination between genes that are up- or downregulated in different conditions. Is the point of this paper to indicate that miRNA downregulation is associated with hypomethylation of the promoter? If so, how do you interpret an inverted correlation in different conditions in this framework? It would seem that the expression of the 'miRNA target genes' is more correlated to the status of their promoter than to the presence of the miRNA.

The author states that this inverse correlation 'is not a discrepancy' but it is actually highlighting the discrepancy that has to be explained rather than brushed off.

Similarly the following statement by the author doesn't seem to be so obvious to me: '0.7 to 0.8, indicate that miRNA-targeting-specific promoter methylation governs at most 50% to 60% of miRNA regulation of target genes. Thus, cell line specific miRNA regulation of target genes can act within the remaining 50% to 40%.' An alternate explanation could be that the methylation doesn't govern the expression status of the genes but reflects it partially (i.e. a degraded messenger could trigger an unknown pathway that would lead the modification of the promoter methylation and stabilize the downregulation at the genomic level.

A similar logical leap is made further down in the text: 'The fact that I observed significant changes in expression of target genes without significant changes in expression of miRNAs that target these genes implicates a role miRNA-targeting-specific promoter hypomethylation.' It could also mean that these genes are regulated transcriptionally without the intervention of miR-548 miRNAs, there are probably other gene groups that are differently expressed in these cell lines.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 04 February 2013

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Wenqian Hu

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This paper reports interesting correlations between the regulation of genes by miRNAs and the methylation status of the miRNA target genes. Although the two processes occur in two different compartments of the cell, these correlations suggest that there may be some potential connections in these two regulatory events of gene expression.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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