Check for updates

RESEARCH ARTICLE

Correlation between miRNA-targeting-specific promoter methylation and miRNA regulation of target genes [version 1; peer review: 1 approved, 1 approved with reservations]

Y-h Taguchi

Department of Physics, Chuo University, Tokyo, Japan

V1 First published: 23 Jan 2013, 2:21
<https://doi.org/10.12688/f1000research.2-21.v1>
 Second version: 27 Mar 2013, 2:21
<https://doi.org/10.12688/f1000research.2-21.v2>
 Latest published: 11 Sep 2013, 2:21
<https://doi.org/10.12688/f1000research.2-21.v3>

Abstract

Background miRNA regulation of target genes and promoter methylation were 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-targeting-specific promoter methylation and miRNA regulation of target genes were computed with the MiRaGE method proposed recently by the author.

Results We found that promoter methylation was miRNA-targeting-specific. In other words, changes in promoter methylation were associated with miRNA binding at target genes. It was also found that miRNA-targeting-specific promoter hypomethylation was related to miRNA regulation; the genes with miRNA-targeting-specific promoter hypomethylation were downregulated during cell senescence and upregulated during cell differentiation. Promoter hypomethylation was especially enhanced for genes targeted by miR-548 miRNAs, which are non-conserved, and 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-targeting-specific promoter hypomethylation was suggested to facilitate gene regulation by miRNAs that are not strongly expressed (e.g., miR-548 miRNAs).

Keywords

cell lines, methylation

Open Peer ReviewApproval Status

	1	2	3
version 3 (update) 11 Sep 2013			
version 2 (update) 27 Mar 2013		view	view
		↑	
version 1 23 Jan 2013	view	view	

1. **Wenqian Hu**, Whitehead Institute for Biomedical Research, Cambridge, MA, USA
2. **Denis Dupuy**, Institut Europeen de Chimie et Biologie, Pessac, France
3. **Pål Sætrom**, Norwegian University of Science and Technology, Trondheim, Norway

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Y-h Taguchi (tag@granular.com)

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

Copyright: © 2013 Taguchi Yh. This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Data associated with the article are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

How to cite this article: Taguchi Yh. **Correlation between miRNA-targeting-specific promoter methylation and miRNA regulation of target genes [version 1; peer review: 1 approved, 1 approved with reservations]** F1000Research 2013, 2:21 <https://doi.org/10.12688/f1000research.2-21.v1>

First published: 23 Jan 2013, 2:21 <https://doi.org/10.12688/f1000research.2-21.v1>

Introduction

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

Among them, the promoter methylation and the 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 rapidly changing. miRNA expression 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., in accordance with 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, to date, 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 cooperative regulation 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 relationship between miRNA-mediated gene regulation and various features of target genes, but they did not consider the methylation status

of target genes 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 miRNA-targeting-specific; 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, the miRNA-targeting-specific promoter methylation is also related to how miRNAs regulate target gene expression. Especially, I find that miR-548 miRNAs target genes 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 by HumanMethylation27 BeadChip (Illumina), which provides an efficient approach for surveying genome-wide DNA methylation profiles. The Human-Methylation27 panel targets CpG sites located within the proximal promoter regions of transcription start sites (TSS). Thus, it was suitable for our purpose. 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 ID: GSM868008¹⁴, GEO ID: GSM739940¹⁷, and GEO ID: GSM375442¹⁸. They were compared to IMR90 promoter methylation profiles, GEO ID: GSM760387 within GEO ID: GSE30653. I also used promoter methylation profiles for IMR90 from GEO ID: GSE31848, which includes GSM868008; these data 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 was 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 the transcription start site (TSS).

mRNA and miRNA expression profiles

In order to compare miRNA-targeting-specific 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 from the site for further analysis and were normalized so as to have a mean of 0 and a variance of 1.

Investigation of miRNA-targeting-specific promoter methylation

In order to infer miRNA-targeting-specific 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 miRNA regulation of target genes. This software 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; however, in this study, I used this method to infer miRNA-targeting-specific 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, usually, the ratio of number of methylated sites to the total number of methylated and non-methylated sites is used to describe promoter methylation levels, I employed a method in which total methylation values were used. I did this 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. For this purpose, depending on the methodology and/or type of deposited data set, I computed *P*-values that describe promoter methylation as follows:

GSM868008

In this case, the 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} represents the scaled values of signal_B (intensity estimated of methylated DNA), which are 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 is the total number of genes considered and M_i is the raw value of signal_B. This signifies that the amount of promoter methylation is scaled so as to have a mean $\langle M_{0i} \rangle$ of zero and standard deviation $\sigma_{M_{0i}}$ of 1. $\exp$ is applied in this instance because I want to consider the amount of methylation rather than the ratio of the amount of promoter methylation. Because *P*-values are computed after the pair of input values are transformed to a logarithmic ratio, by substituting 1 in the control dataset and an exponential value in the treatment dataset based on raw values results in the usage of raw values as differential expression/promoter methylation (see below).

GSE30653

In this case, the 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 takes 1 only when $M_i = 0$; otherwise, it takes on 0, so as to avoid infinite values after transformation to the logarithmic ratio.

GSM739940

In this case, the 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 is the ratio of methylated sites to unmethylated sites,

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

where U_i is the signal from unmethylated sites (signal_A) and C is the regulation constant, which typically takes on the value of 100. Since only β values were deposited in the public datasets used, we could not avoid using them; however, the correlation with target gene miRNA regulation was substantially decreased. An explanation for this is noted above.

GSM375442

In this case, the 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 $\max(M_i)$ is the maximum value of M_i and M_i is computed in this case as follows:

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

where y_j , $0 \leq y_j \leq 100$ is the percentage of methylation at site j , which was computed by 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

Here are the command line inputs used for generating the 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. We also assumed that `R`²² was installed but the part executed

by `R` can be performed by any other alternative script 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 sites, y_j , which was explained above.

Inference of miRNA-targeting-specific promoter methylation/miRNA regulation of target genes by MiRaGE method

The inference of miRNA-targeting-specific 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 our 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 $\exp(x_j)$ can be used instead of x_j , in which case I get

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

When computing P -values that reject 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 any other miRNAs, I compute

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

where $P[A < (>) B]$ is P -values computed by statistical tests when two set 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, judging $A < (>) B$ depends upon the selected statistical test used. G_m represents the set of genes targeted by miRNA m and G'_m is 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 line 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-targeting-specific promoter methylation and miRNA regulation of target genes

I have used two types of P -values, $P_{m:\text{methy}}^{(< >)}$ which corresponded to the miRNA-targeting-specific promoter methylation, and $P_{m:\text{regul}}^{(< >)}$ which corresponded to the miRNA regulation of target genes of miRNA m . When $P_{m:\text{methy}}^{(< >)}$ is 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 $\rho \neq 0$. $\rho[a, b]$ is the Pearson's correlation coefficients between a and b and $\text{rank}(x_m)$ is the rank order of x_m among $\{x_m\}$. $P_{m:\text{methy}}^{(< >)}$ where $z \in \{\text{methy}, \text{regul}\}$ is either $P_m^<$, $P_m^>$, $1 - P_m^<$ or $1 - P_m^>$. Thus, there are $4 \times 4 = 16$ possible combinations of $P_{m:\text{methy}}^{(< >)}$ and $P_{m:\text{regul}}^{(< >)}$. ρ s of Kolmogorov-Smirnov test and $\rho_{\log}^{\text{Pearson}}$ s for all tests can change when $P^{(< >)}$ is replaced with $1 - P^{(< >)}$ because $P^{(< >) \neq 1 - P^{(< >)}$ for Kolmogorov-Smirnov test and $\log(P^{(< >)}) \neq \log(1 - P^{(< >)})$ for all tests. Thus, optimal combinations with 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 differentially expressed between undifferentiated

and differentiated cell lines were selected based on two criteria before being subjected to further analyses;

- Absolute differential expression $|x_{\text{diff},i} - x_{\text{undiff},i}| > \Delta x_e$, where $x_{\text{diff},i}$ and $x_{\text{undiff},i}$ are the normalized expression of gene i of differentiated and undifferentiated cell lines, respectively. Δx_e is the threshold value that can 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_e 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 should be 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), miRNA names 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-targeting-hypomethylated promoters

miRNAs were ranked based on $P_m^>$ in each of 283 samples in GSE30653, after excluding 12 control samples. Each miRNA was ranked based within each sample, and then, the cumulative rank was determined for 271 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 order summed up over three statistical tests.

Results and discussion

Based on the inference from the data produced by 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 hypomethyl-

Table 1 miRNA-targeting-specific promoter hypomethylation.

Amount of methylation			
Statistical tests	Number of miRNAs		
	Not significant	Significant	
<i>t</i> -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	
<i>t</i> -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 271, 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 file 1. Cell line names can be also identified as column names of the additional file 1.

ated promoters. Given that promoter methylation patterns do not change drastically between certain cell types, the amount of miRNA-targeting-specific promoter methylation is also highly cell-type-independent. Mean correlation coefficients of $P_m^>$ range 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 see if miRNA-targeting-specific promoter methylation was genuinely related to miRNA regulation of target genes, I compared P -values of miRNA-targeting-specific promoter methylation to P -values of miRNA regulation of target genes

Table 2 Mean correlation coefficients between P -values of miRNA-targeting-specific promoter hypomethylation of samples. Mean Pearson's correlation coefficients of $P_m^>$ between pairs of cell lines.

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

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 3), despite the fact that correlation

Table 3 Correlation coefficients between P -values of miRNA-targeting-specific promoter hypomethylation and miRNA regulation of target genes. * Indicates $< 2.2 \times 10^{-16}$.

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

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.

To confirm that the significant correlation noted above between miRNA-targeting-specific 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 by another group of researchers¹⁸ (Table 4). 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 conditions.

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 5).

Thus, I conclude that miRNA-targeting-specific promoter methylation is not an artifact, but a genuine biological process. One may doubt this conclusion given the finding that miRNA-targeting-specific 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-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%. In actuality, as can be seen in Table 3, the correlations between miRNA-targeting-specific 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-targeting-specific promoter methylation partially governs cell line dependent miRNA regulation of target genes is not a discrepancy.

In order to better understand the biological significance of our findings regarding miRNA-targeting-specific 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 6; here, I define miRNAs called “miR-548” 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

Table 4 Evaluation of the correlation between miRNA-targeting-specific promoter methylation and miRNA regulation of target genes.

Statistical test	ρ_{Pearson}	ρ_{log}	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-targeting-specific promoter methylation and miRNA regulation of target genes during cell senescence shown in Table 3 were evaluated by a comparison with the results produced by different array design (GSM868008), distinct research group, alternative measures of promoter methylation (GSM739940), and NGS (GSM375442). Independent of these factors, miRNA-targeting-specific promoter methylation was found to be always positively correlated to miRNA regulation of target genes (i.e., genes with miRNA-targeting-specific promoter hypomethylation were downregulated during cell senescence).

Table 5 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 is 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 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).

Table 6 Evaluation of miR-548 miRNAs targeting genes with miRNA-targeting-specific promoter hypomethylation.

Statistical Test	Number of miR-548 miRNAs		
	Within top 100 ranked	Total number	Mean rank
t 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, $P = 2 \times 10^{-35}$.

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 amount 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 millions 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^{25,26}.

In this study, here 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.

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^{27,28}. Since it is also suggested that they originate from transposable

elements (TEs), miR-548 miRNAs exhibit high sequence similarities while maintaining significant sequence diversity²⁷.

During senescence processes of IMR90 and MRC5 cell lines, the target genes of miR-548 miRNAs were significantly down-regulated (Table 7), whereas during differentiation of the BG02 cell line, the target genes of miR-548 miRNAs were significantly upregulated (Table 7). 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.

Conclusions

Figure 1 schematically summarises the principal findings of this study. To the best of my knowledge, this is the first time that miRNA-targeting-specific promoter methylation in many types of cell lines have been observed (Tables 1 and 2). miRNA-targeting-specific hypomethylation was correlated (Tables 3 and 4) with regulation of miRNA target genes, which had a reciprocal relationship with miRNA expression (Table 5); genes with miRNA-targeting-specific hypomethylated promoters were downregulated during cell senescence and upregulated during differentiation. I also found that genes with miRNA-targeting-specific hypomethylated promoters were specific targets of miR-548 miRNAs (Tables 6 and 7). Regulation of target genes by miR-548 miRNAs, which were typically expressed at low levels are likely the result of miRNA-targeting-specific promoter hypomethylation.

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

Table 7 Ranking of miRNA regulation of target genes.

Cell lines	Target genes	Number of miR-548 miRNAs			P-values
		Within top 100 ranked	Total number	Mean rank	
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 weakly correlated to miRNA expression and miRNA regulation of target genes (Table 5). Given that the total number of miRNAs considered was 1921, the values for IMR90, MRC5, and BG02 cell lines are highly significant.

Figure 1 Schematic representing the principal findings of the present study together as well as those reported previously. Promoters are methylated in a miRNA-targeting-specific manner (Tables 1 and 2). Genes with miRNA-targeting-specific hypomethylated promoters are downregulated during cell senescence and upregulated during cellular differentiation (Tables 3 and 4), whereas miRNAs that target genes with hypomethylated promoters were upregulated during cell senescence^{17,18} and downregulated during differentiation (Table 5). miR-548 miRNAs, which are expressed at low levels, were suggested to regulate target genes with the assistance of miRNA-targeting-specific promoter hypomethylation (Tables 6 and 7).

Competing interests

No relevant competing interests disclosed.

Author's contributions

Conceived and designed the experiments: YHT. Analyzed the

data: YHT. Wrote the paper: YHT.

Acknowledgements

The author thanks the anonymous reviewer¹⁶ who suggested the investigation of the promoter methylation of miRNA target genes. This study was supported by KAKENHI (23300357).

Additional Files

Additional file 1 — Full list of *P*-values from the analysis of miRNA-targeting-specific 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. F1000 Research. Y-h Taguchi Retrieved 15:55, Jan 10, 2013 (GMT)

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

Additional file 4 — Full list of *P*-values from the analysis of miRNA-targeting-specific 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. F1000 Research. Y-h Taguchi Retrieved 15:55, Jan 10, 2013 (GMT)

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

Additional file 2 — Full list of *P*-values from the analysis of miRNA-targeting-specific 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. F1000 Research. Y-h Taguchi Retrieved 15:55, Jan 10, 2013 (GMT)

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

Additional file 5 — Full list of *P*-values from the analysis of miRNA-targeting-specific 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. F1000 Research. Y-h Taguchi Retrieved 15:55, Jan 10, 2013 (GMT)

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

Additional file 3 — Full list of *P*-values from the analysis of miRNA-targeting-specific 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. F1000 Research. Y-h Taguchi Retrieved 15:55, Jan 10, 2013 (GMT)

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

Additional file 6 — Full list of *P*-values from the analysis of miRNA-targeting-specific 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. F1000 Research. Y-h Taguchi Retrieved 15:55, Jan 10, 2013 (GMT)

<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, Galiova G, Kozubek S. 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, Strengman E, Janson E, Sommer IE, Kahn RS, Ophoff RA: The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. *PLoS ONE* 2009, **4**(8): e6767.
- Berardi E, Poes M, Thorrez L, Sampaioles M. microRNAs in ES Cell Differentiation. *Am. J. Physiol. Heart Circ. Physiol.*, in press.
- Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: A new source of biomarkers. Elsevier 2011.
- 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, Chang HY, Segal E. Systematic functional characterization of cis-regulatory motifs in human core promoters. *Genome Res.* 2008, **18**(3): 477–488.
- Saito T, Sffitrom 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, Tran H, Harness JV, Slavin I, Garitaonandia I, Muller FJ, Wang YC, Boscolo FS, Fakunle E, Dumevska B, Lee S, Park HS, Olee T, D'Lima DD, Semechkin R, Parast MM, Galat V, Laslett AL, Schmidt U, Keirstead HS, Loring JF, Laurent LC. 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 and Disease*, in press.
- 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 2012: 441–446.
- Ohm JE, Mali P, Van Neste L, Berman DM, Liang L, Pandiyan K, Briggs KJ, Zhang W, Argani P, Simons B, Yu W, Matsui W, Van Criekinge W, Rassool FV, Zambidis E, Schuebel KE, Cope L, Yen J, Mohammad HP, Cheng L, Baylin SB. 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, Gore A, LeProust EM, Antosiewicz-Bourget J, Egli D, Maherali N, Park IH, Yu J, Daley GQ, Eggan K, Hochedlinger K, Thomson J, Wang W, Gao Y, Zhang K. 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, Song S, Nelson A, Tan Y, Mathieu J, Darby C, Blau CA, Ware C, Peters G, Miller DG, Shen L, Cleary MA, Ruohola-Baker H. Characterization of microRNAs involved in embryonic stem cell states. *Stem Cells Dev.* 2010, **19**(7): 935–950.
- Yoshizawa M, Taguchi YH, Yasuda J. 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, [http://www.R-project.org/]. [ISBN 3-900051-07-0].
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B* 1995, **289**–300.
- Lu TP, Lee CY, Tsai MH, Chiu YC, Hsiao CK, Lai LC, Chuang EY. miRSystem: An Integrated System for Characterizing Enriched Functions and Pathways of MicroRNA Targets. *PLoS ONE* 2012, **7**(8): e42390.
- Marasa BS, Srikantan S, Martindale JL, Kim MM, Lee EK, Gorospe M, Abdelmohsen K. 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, Chen S, Zhang Y, Zhi F, Wang J, Li L, Zhou X, Li N, Pan H, Zhang J, Zen K, Zhang CY, Zhang C. Identification of seven serum microRNAs from a genome-wide serum microRNA expression profile as potential noninvasive biomarkers for malignant astrocytomas. *Int J Cancer* 2012.
- Lin S, Cheung WK, Chen S, Lu G, Wang Z, Xie D, Li K, Lin MC, Kung HF. 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. Genome-wide Analysis of mir-548 Gene Family Reveals Evolutionary and Functional Implication. *J. Biomed. Biotechnol.*, in press.

Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 06 February 2013

<https://doi.org/10.5256/f1000research.1183.r751>

© 2013 Dupuy D. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Denis Dupuy

Institut Europeen de Chimie et Biologie, Pessac, France

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

<https://doi.org/10.5256/f1000research.1183.r748>

© 2013 Hu W. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Wengian Hu

Center for RNA Molecular Biology, Whitehead Institute for Biomedical Research, Cambridge, MA, USA

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.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research