



Ten-eleven translocation (Tet) and thymine DNA glycosylase (TDG), components of the demethylation pathway, are direct targets of miRNA-29a



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ABSTRACT

The ten-eleven translocation family of proteins (Tet1/2/3, Tets) converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which can be further oxidized and repaired by thymine DNA glycosylase (TDG), to influence gene transcription in embryonic and adult tissues. However the mechanisms of how Tets and TDG levels are regulated are unknown. We show that miR-29 can directly regulate Tet1-3 and TDG mRNA levels through binding to their 3'UTRs. miR-29 mimic decreases global 5hmC levels, a hallmark of Tet activity. Moreover, the mRNA levels for Tet3 and TDG are inversely correlated with the levels of miR-29 in aged mouse aorta implying that aging may affect methylation patterns via miRNA. In summary, our data show that Tets and TDG are direct targets of miR-29 and unravel a novel regulatory role for this miRNA in epigenetic DNA demethylation pathways.

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1. Introduction

DNA methylation is a major epigenetic modification in the eukaryotic genome that regulates gene expression during many biological processes. Although the DNA methyltransferase (DNMT) family of enzymes establish and maintain DNA methylation, the ten-eleven translocation (Tet) family of enzymes (Tet1, Tet2, and Tet3) are implicated in DNA demethylation and epigenetic control of gene expression [1–3]. The Tet enzymes have dioxygenase activity and can convert 5-methyl cytosine (5mC) to 5-hydroxymethylC (5hmC), 5-formyl cytosine (5fC), and 5-carboxylcytosine (5caC) [4,5]. These 5mC derivatives can be recognized and removed by the base excision repair machinery involving thymine DNA glycosylase (TDG) [4,6] resulting in demethylation on once methylated cytosines. In addition to Tet/TDG role in active demethylation, Tets also fine-tune epigenetic processes by binding to NANOG and synergistically enhance the efficiency of reprogramming [7] or binding to O-GlcNAc transferase to influence histone methyltransferase GlcNAcylation [8]. TDG, on the other hand, has been shown to

associate with DNMTs to maintain normal methylation patterns [9], or bind to transcription coactivators CBP/p300 for transcription modulation and base repair [10].

In the past decade, microRNAs (miRNAs) have emerged as important regulators of gene expression robustness. miRNAs predominantly target the 3'UTR of mRNAs, either destabilize the mRNA transcript or interfering with its translation into protein. miRNA can influence the epigenetic code by regulating histone acetylase and methyltransferase [11–13] and by targeting transcription factors which may control Dnmt transcription [14]. Recent evidence suggests that the miR-29 family of miRNAs can influence gene methylation patterns since miR-29 can bind to 3'UTR of the methyltransferases, DNMT3a and 3b, to maintain normal DNA methylation patterns [15]. However, whether miRNAs regulate the pathway leading to DNA demethylation has not been studied. Here, we show that miR-29 can also control demethylation reactions by targeting the 3'UTRs of Tet-1,-2 and -3 and TDG and repressing their mRNA levels. miR-29 mimics decrease Tet and TDG mRNA levels and global 5hmC levels, while miR-29 inhibition increase their mRNA and 5hmC levels. These data suggest that miR-29 regulates two key emerging players in DNA demethylation and epigenetic control supporting a novel role of miRNAs in epigenetic regulation of gene expression.

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2. Materials and methods

2.1. Cell culture and miRNA treatment

Human dermal fibroblasts (HDF) and vascular smooth muscle cells (VSM) were obtained and cultured as described [16]. 293 HEK cells were grown in DMEM with 10% fetal bovine serum. Cells were treated with miRIDIAN miRNA mimic, hairpin inhibitors or corresponding controls (Dharmacon, Chicago, IL) at 60 nM for 15 h unless specified otherwise, then were grown in complete media for 48 h before harvest for RNA or protein studies. siRNA against human Tet3 was purchased from QIAGEN and transfected cells at 50 nM with RNAiMax (Invitrogen).

qPCRTotal RNA, including miRNA, was isolated by miRNAeasy isolation kit (Qiagen). After reverse transcription, qPCR was performed using Bio-Rad real-time thermal cycler and power SYBR green master mix (Applied Biosystem). mRNA levels were normalized by those of GAPDH and control levels.

2.2. Western blotting

Nuclear extracts were harvested using NE-PER nuclear extraction kit (Thermo Scientific) and equal lysates (10 µg) were loaded onto 7% SDS-PAGE. Western membranes were probed with a polyclonal antibody recognizing human Tet3 (Abicodex). Hsp90 levels were used to normalize protein loading.

2.3. Luciferase 3'UTR luciferase reporter assay

3'UTRs of human Tet1-3 at indicated length were cloned behind Renilla luciferase gene in psiCHECK2TM dual luciferase reporter vector (Promega). Cells were transfected with 60 nM miRNA together with 0.1 µg luciferase vector for 24 h. Luciferase signals were normalized by signals from a constitutively expressed firefly luciferase gene and signals from control treated groups. Mutations in predicted miR-29 target sites were generated using quikchange lightning site-directed mutagenesis kit (Agilent Technologies).

2.4. 5hmC dot-blot

1 µg genomic DNA were denatured with 0.1N NaOH and spotted on GE/Amersham Hybond-N+ membrane using 96-vacuum apparatus. After UV cross-linking, membranes were probed with 5hmC antibody (Active Motif). To ensure equal spotting of total DNA on the membranes, the same blot was stained with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5).

2.5. 3'RACE

3'RACE was performed using FirstChoice® RLM-RACE kit (Invitrogen) according to manufacturer instructions. Briefly, total RNA were isolated and converted to cDNA using 3'RACE adaptor primer. PCR were performed using gene specific and adaptor primers, and products analyzed by agarose electrophoresis.

2.6. Microarray

Total RNA from HDF cells after 48 h treatment with 90 nM miR-29a inhibitor or control inhibitor were isolated and hybridized to GeneChip Human Gene 1.0 ST Array that covers 28,869 genes (Affymetrix) at Yale center for Genome analysis. Microarray analyses were performed as described [17].

2.7. Statistical analysis

Comparisons between two groups were by unpaired student t test. Statistical analyses were performed using Prism 4 software (GraphPad). P values were two tailed and values <0.05 were considered to be statistical significant.

3. Results and discussion

Our previous results have shown that anti-miR 29a treatment of human dermal fibroblast (HDF) and vascular smooth cells (VSM) markedly upregulates elastin mRNA levels and this anti-miR reduces levels of miR-29a, -b and -c [16]. To examine the broad

miR-29a 5'-UAGCACCAUCUGAAAUCGGUUA-3'	
Tet1: NM_030625.2	-3 283 400 509
Tet1 3'UTR (509bp)	5'-UGA..... UGGUGCU u..... UGGUGCU u.....AAUAAA...-3'
Tet1-1/2mt	5'-UGA.....UG <u>C</u> AGCUu.....UG <u>C</u> AGCUu.....AAUAAA...-3'
Tet2: NM_001127208.2	-3 447 596 811
Tet2 3'UTR (811bp)	5'-UGA..... UGGUGCU g..... UGGUGCUAAAUAAA...-3'
Tet2-1/2mt	5'-UGA.....UG <u>C</u> AGCUg.....UG <u>C</u> AGCUA.....AAUAAA...-3'
Tet2V2: NM_017628.3	-3 580 597
Tet2V2A1 3'UTR (580bp)	5'-UAA.....AAUAAA...-3'
Tet2V2A2 3'UTR (597bp)	5'-UAA.....AAUAAA...-3'
Tet3: NM_144993 Tet3 3'UTR (1963bp)	-3 53 1070 1084 1101 1471 1963
Tet3-2/3mt	5'-UAG... UGGUGCU u... UGGUGCU c... UGGUGCU g... UGGUGCU g... UGGUGCU u...AAAAA...-3'
Tet3-3/4mt	5'-UAG...UGGUGCUu...UG <u>C</u> AGCUc...UG <u>C</u> AGCUg...UGGUGCUg...UGGUGCUu...AAAAA...-3'
Tet3-3/4mt	5'-UAG...UGGUGCUu...UGGUGCUc...UG <u>C</u> AGCUg...UG <u>C</u> AGCUg...UGGUGCUu...AAAAA...-3'
TDG: NM_003211.4	-3 878 1064 1754
TDG 3'UTR (1754bp)	5'-UAA..... UGGUGCU u..... UGGUGCUAAAUAAA...-3'
TDG-1/2mt	5'-UAA.....UG <u>C</u> AGCUu.....UG <u>C</u> AGCUA.....AAUAAA...-3'

Fig. 1. Predicted binding sites for miR-29 in the 3'UTRs of Tet genes and TDG. The length of the 3'UTR is listed with target sites for miR-29a in bold. Mutants generated are listed below the WT sequence and mutations indicated by nucleotides underlined.

effects of miR-29 inhibition on global gene expression patterns, HDF were treated for 48hr with anti-miR 29a and the levels of gene expression analyzed by Affymetrix microarrays. Treatment with anti-miR 29a significantly increased the expression levels of only 33 transcripts implying that endogenous miR-29 in HDF represses a relatively small set of transcripts (Fig. 1 and GEO access), suggesting they are likely to be physiological targets of miR-29. Among the genes elevated with anti-miR29 were Tet1 and Tet3, whose levels were increased by more than 50% after miR-29 inhibitor treatment. Indeed, in the 3'UTR there are two potential miR-29 target sequences in Tet-1 and Tet-2 and five potential sites in Tet-3 (Fig. 1). To validate the microarray data, three different donors of HDF and VSM were analyzed by qPCR. Indeed, miR-29a mimics decreased the levels of three Tets and miR-29a inhibition increased all three Tets in HDF (Fig. 2A, left) and markedly upregulated Tet3 in VSM (Fig. 2A, right). qPCR assays using primers that do not distinguish between Tet2 variants showed that the miR-29 inhibitor had no effect on Tet2 mRNA levels in VSM (Fig. 2A, right).

Computational analysis predicts several miRNAs other than miR-29 may have binding sites in the 3'UTR of Tet transcripts, including miRNAs -17, -101 and -26. However, among the miRNAs tested, only the miR-29a mimic consistently decreased Tet1 and Tet3 transcripts

while the miR-29a inhibitor increased Tet1 and Tet3 levels consistent with a pattern of direct targeting (Fig. 2B, left and right panels). The effects of miR-17, -101 and -26 were variable in multiple experiments. Deeper analysis of Tet2 revealed two forms of the Tet2 transcript, a Tet2 variant 1 (Tet2) with consensus miR-29a site or a shorter form Tet2 variant 2 (Tet2V2) with alternative polyadenylation sites (named Tet2V2A1 and Tet2V2A2) lacking putative binding sites for miR-29a. Indeed, qPCR experiments using primers to distinguish these two variants showed only Tet2, but not Tet2V2, has the potential to be directly targeted by miR-29a (Fig. 2B, middle panels). 3'RACE studies as well as careful examinations of 3'UTR sequences of Tets suggested that HDF cells tend to use alternative polyadenylation signals and produce Tet transcripts with shorter 3'UTRs which still contain regions for miR-29 binding (Fig. 1 and Supplementary Fig. 1). Transcripts can be processed at one or multiple poly (A) signals to generate 3'UTR of varied length, depending on the cell type and circumstances [18,19]. The number of binding sites seems to influence the sensitivity of Tet transcripts by miR-29 as Tet3 has the most predicted sites and is consistently to be the most significantly regulated Tet by miR-29 in HDF and VSM (Fig. 2A).

In order to examine whether Tets are direct targets of miR-29, the 3'UTRs of selected length for Tet1, 2 and 3 (Fig. 1) were cloned

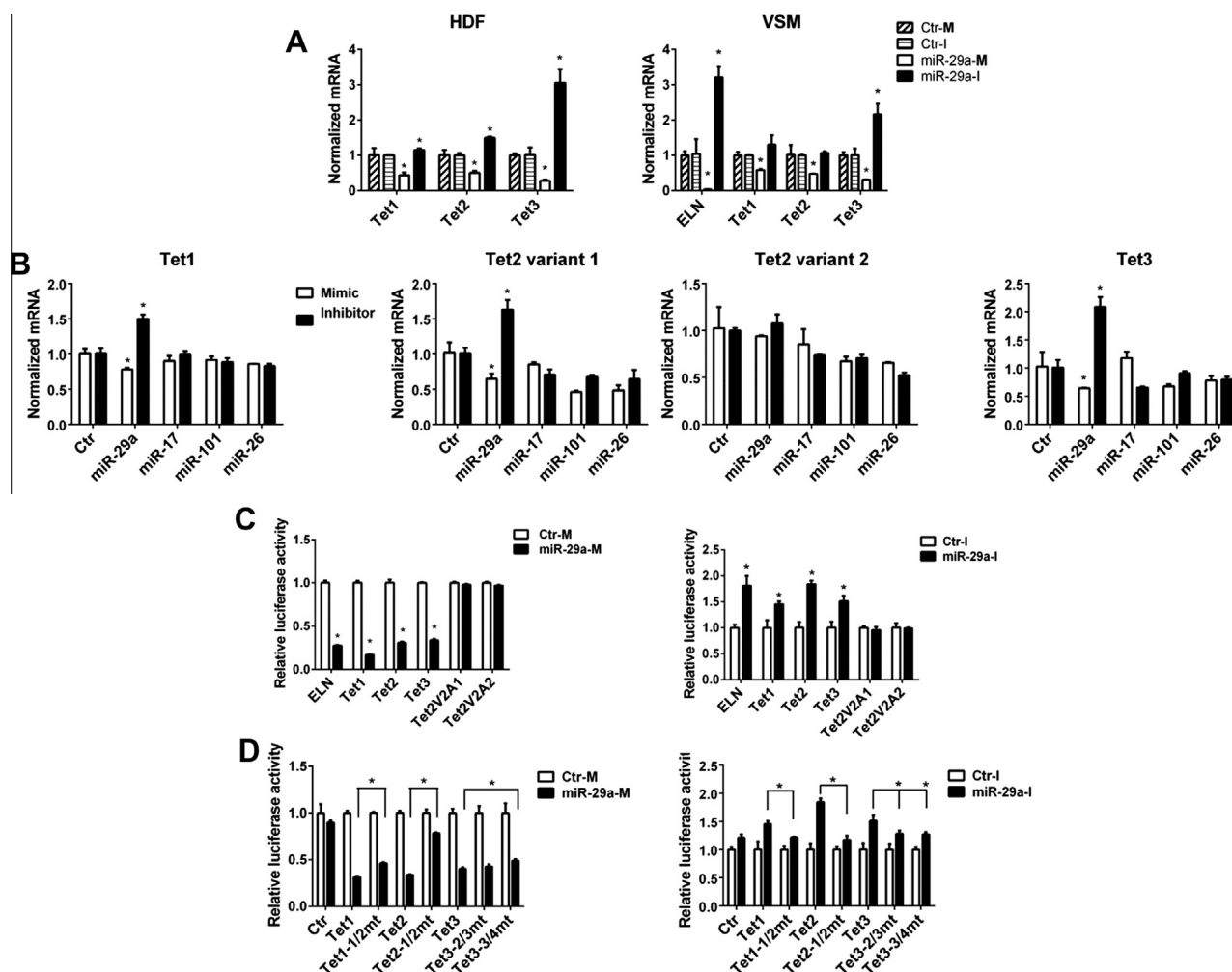


Fig. 2. Tet1-3 are direct targets of miR-29a. (A) HDF or VSM were treated with miR-29a mimic (M) or inhibitor (I) or controls (Ctr-M or Ctr-I) for 48 h and mRNA were analyzed for Tet expression. (B) HDF were treated with indicated miRNA mimic/inhibitor for 48 h, and Tet mRNA levels were analyzed. (C) HEK 293 cells (left panel) or HDF (right panel) were co-transfected with luciferase reporter constructs for the 3'UTRs of ELN, Tet1-3 and Tet2 variants and control mimics (Ctr-M), miR-29-M or miR-29a-I. The Tet2V2A1 and Tet2V2A2 constructs lack consensus sites for miR-29 as shown in Fig. 1. (D) HEK 293 cells (left panel) or HDF (right panel) were co-transfected with WT or mutant luciferase reporter constructs and Ctr-M, miR-29-M, Ctr-I or miR-29-I. Tet mutants (Tet1-1/2, Tet2-1/2, Tet3-2/3 and Tet3-3/4) are depicted in Fig. 1. Data are representative of 3 independent experiments. In all cases, data are mean \pm SEM, * p < 0.05 vs. control or otherwise indicated.

into dual luciferase reporter constructs. Transfection with the control luciferase reporter without a cloned in 3'UTR (empty vector) did not affect luciferase activity (Fig. 2D). miR-29a mimic treatment decreased luciferase activities by more than 50% using the 3'UTR containing a well described miR-29 target, ELN [16,20] and also reduced Tet1, Tet2 as well as Tet3, but not in the two Tet2V2 constructs (Fig. 2C, left). Likewise, miR-29a inhibitor increased luciferase activities in constructs containing ELN, Tet1, Tet2 and Tet3, but not in Tet2V2 (Fig. 2C, right). When two nucleotides in miR-29 binding sites in Tet1 and Tet2 3'UTR were mutated from "GU" to "CA" (see Fig. 1 for construct), the effects of miR-29a mimic or inhibitor on these 3'UTRs were significantly attenuated (Fig. 2D). However, there are five putative miR-29 binding sites in the first 1963 bp of Tet3 3'UTR and mutating sites 2 and 3 (Tet3-2/3mt) or sites 3 and 4 (Tet3-3/4mt) can only marginally rescue the effects of miR-29a mimic or inhibitor, suggesting the involvement of other sites or cooperativity between the sites (Fig. 2D).

In order to examine Tet protein levels and potential functions of miR-29, nuclei were isolated from HDF treated with miR-29 mimic or inhibitor. We were unsuccessful in reproducibly detecting Tet1 or Tet2 protein levels with commercially available antibodies in isolated nuclear extracts. However, miR-29a mimic decreased while miR-29a inhibitor partially increased Tet3 protein levels

(Fig. 3A). Treatment with a siRNA against Tet3 decreased Tet3 mRNA levels in both control and miR-29a inhibitor treated groups, but had no effect on Tet1 or Tet2 mRNAs (Fig. 3B). This Tet3 siRNA also decreased Tet3 protein levels, similar to that observed with miR-29a mimic (Fig. 3C). To examine if miR-29 can influence Tet activity, we assayed the Tet-dependent conversion of 5mC to 5hmC. To test this, genomic DNA was isolated after miR-29 mimic/inhibitor treatment for 48 h and the levels of 5hmC determined by dot blotting. As seen in Fig. 3D, knockdown of Tet3 as well as treatment with the miR-29a mimic modestly decreased global 5hmC levels, while the miR-29a inhibitor modestly increased global 5hmC levels (as quantified in the right panel). The inability to observe a complete loss or gain of 5hmC may be due to other Tet isoforms that lack target sites for miR-29, multiple Tet isoforms, and competing methylation reactions. In addition, there may be kinetic lag between the effects of miRNA mimics and inhibitors on mRNA, protein and integrated function. Tets are the only identified enzymes having abilities to oxidize 5mC to 5hmC [21], so the inverse correlation between miR-29 and 5hmC levels strongly support our hypothesis that miR-29 regulates Tet directly. Tet3 may be the major contributing Tet in HDF since knocking down Tet3 alone achieved a similar effect in reducing 5hmC as miR-29 mimic treatment. 5hmC can be further oxidized

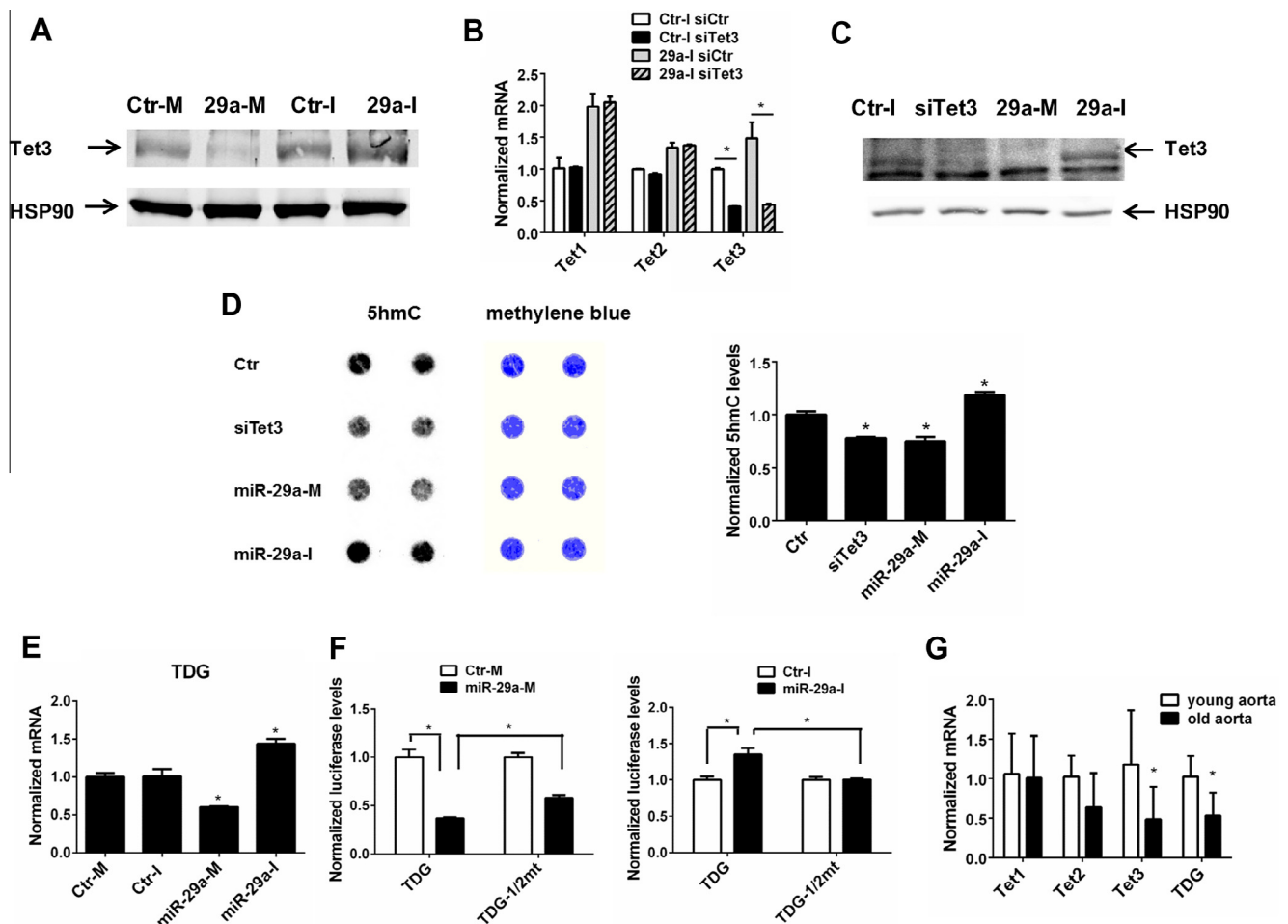


Fig. 3. miR-29 regulates Tet3 protein, global 5hmC and TDG levels. (A–D) HDF were treated with indicated miRNA, siRNA against Tet3 (siTet3) or in certain cases both for 48 h. Nuclear extracts were isolated and Western-blotted for Tet3 protein levels (A and C); mRNA were examined by qPCR for Tet3 knock-down efficiency and specificity (B); total genomic DNA were isolated and dot-blotted with antibodies for 5hmC, which are normalized by methylene blue staining for total DNA (D). (E) Total RNA from miRNA-treated VSM were isolated and analyzed for TDG mRNA. (F) Left, HEK 293 cells were transfected with TDG luciferase reporter constructs and Ctrl-M or miR-29-M; right, HDF cells were transfected with TDG luciferase reporter constructs and Ctrl-I or miR-29-I. (G) RNA from old (22 months) and young (2 months) mouse (C57BL/6) aorta ($n = 6$) were analyzed for Tet and TDG expression. Data are representative of 3 independent experiments. In all cases, data are mean \pm SEM, $^*p < 0.05$ vs. control or otherwise indicated.

by Tets to 5fC and 5caC, which are excised by TDG and eventually repaired [4,22]. Loss of TDG is associated with aberrant methylation pattern and accumulation of 5fC and 5caC at gene regulatory elements [23,24]. Interestingly, TDG 3'UTR also contains two miR-29 binding sites and we identified that miR-29 mimic decreased TDG mRNA by 40%, while miR-29 inhibitor increased TDG mRNA by 43.7% in VSM (Fig. 3E, similar data in HDF not shown). These effects are mediated by miR-29 binding to TDG 3'UTR because point-mutations of both predicted binding sites significantly reversed the repressive effect of miR-29 on luciferase activity (Fig. 3F). Therefore, our data demonstrated a novel regulatory role of miR-29 in active DNA demethylation, targeting both Tets and TDG.

TDG can also bind to DNA methylation enzymes to maintain normal methylation patterns [9]. miR-29 members were shown to target to Dnmt3a and 3b through complementary binding to their 3'-UTRs and enforced expression of miR-29 can restore normal activities of Dnmts and normal DNA methylation pattern in certain cancer cell lines [15]. We have verified that miR-29 can regulate Dnmt3a and 3b in both HDF and VSM (data not shown), but the functional consequences in vascular cells remain to be determined. DNA methylation and demethylation are very dynamic and fine-tuned events and can coexist in the same promoter; i.e. – 5hmC and 5mC can be both enriched in certain promoters in embryonic stem cells [3,25]; Dnmts and Tets can both increase at intermediate reprogramming stage of induced pluripotent stem (iPS) cells generation, leading to increased production of both base modifications [26]. However, the overall effects of miR-29 on DNA methylation/demethylation patterns remain largely unknown and may be tightly controlled by effectors regulating miR-29 expression. Previous work has shown that miR-29 levels are low during embryogenesis, but increase into adulthood and during aging [27–29]. As a corollary to this both Tet3 and TDG levels were significantly lower in aged mouse aortae (22 months) when compared to young mouse aortae (2 months; Fig. 3G), suggesting that Tet and TDG may be reciprocally regulated by miR-29 in vivo.

In the past several years, intense research delineating the exciting range of functions of Tet/TDG in epigenetic demethylation reactions has shown their roles in crucial cellular events such as stemness, lineage determination, cell type specific functions and aging. Our data has defined an important regulatory role of miR-29 in modulating these key emerging players may yield insights into processes and diseases associated with alterations DNA methylation/demethylation.

Microarray GEO access

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=dvyjxyeyaygyctk&acc=GSE45564>.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.06.082>.

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