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Upregulation of *PHLDA2* in *Dicer* knockdown HEK293 cells

Kai-Fu Tang [a,*], Yan Wang [d], Pengfei Wang [b], Min Chen [a], Yao Chen [a], Huai-Dong Hu [a], Peng Hu [a], Bo Wang [c], Wenjie Yang [e], Hong Ren [a,*]

[a] *Key Laboratory of Molecular Biology for Infectious Diseases of State Ministry of Education, The second Affiliated Hospital, Institute for Viral Hepatitis, Chongqing University of Medical Sciences, Chongqing 400010, PR China*
[b] *Department of Emergency, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, PR China*
[c] *Department of Endocrinology, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, PR China*
[d] *The Institute of Psychology, Chinese Academy of Sciences (CAS), Beijing 100101, PR China*
[e] *Department of Epidemiology, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA 70112, USA*

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Abstract

It has been reported that RNAi-dependent chromatin silencing in vertebrates is not restricted to the centromeres. To address whether RNAi machinery could regulate the chromatin structure of imprinted genes, we knocked down *Dicer* in HEK293 cells and found that the expression of *PHLDA2,* one of the several genes in the imprinted gene domain of 11p15.5, was specifically upregulated. This was accompanied by a shift towards more activated chromatin at *PHLDA2* locus as indicated by change in H3K9 acetylation, however, the methylation state at this locus was not affected. Furthermore, we found that *PHLDA2* was downregulated in growth-arrested HEK293 cells induced by either serum deprivation or contact inhibition. This suggests that *PHLDA2* upregulation might be a direct result of *Dicer* depletion rather than the consequence of growth arrest induced by *Dicer* knockdown. Considering the reports that there is consistent placental outgrowth in *PHLDA2* knockout mice and that *PHLDA2* overexpression in mice causes growth inhibition, we speculate that *PHLDA2* may be a candidate for contributing to the reduced growth rate of *Dicer*-deficient cells and the very early embryonic lethality in *Dicer* knockout mice.

Keywords: *PHLDA2*; *Dicer*; Genomic imprinting; Embryonic lethality; Growth arrest

1. Introduction

Genomic imprinting is a form of non-Mendelian gene expression, in which the expression of an allele depends on its parent of origin. Some imprinted genes are expressed from the maternally inherited chromosomes and others from the paternally inherited chromosomes [1]. Imprinted genes have diverse functions, notably including the regulation of growth and metabolism [2]. A number of human disorders are associated with increased or insufficient dosage of imprinted gene products. These disorders fall into two main categories: neurological disorders and disorders of growth and development. Of these, the best characterized are the Prader–Willi (PWS), Angelman Syndromes (AS) and Beckwith–Wiedemann Syndrome (BWS) [3].

Imprinted genes are often clustered in large domains. Their allelic repression is regulated by 'imprinting control regions' (ICRs), which are methylated on one of the two parental alleles. Chromatin structure, including DNA methylation and histone modifications, is important for the somatic maintenance of imprinting [4]. RNA interference (RNAi) is a natural process of eukaryotic cells by which double-stranded RNA initiates and directs the degradation of homologous mRNA [5]. RNAi machinery is also involved in the regulation of chromatin structure, mutants in RNAi machinery display a loss of heterochromatin at the centromeres, a derepression of transcription of centromeric repeats and an aberrant accumulation of complementary transcripts from centromeric heterochromatic repeats [6–10].

* Corresponding authors. 74# Linjiang Road, The Institute of Viral Hepatitis, The Second Affiliated Hospital, Chongqing Medical University, Chongqing 400010, PR China. Tel.: +86 13452037049; fax: +86 23 63822696.
E-mail addresses: tangkaifu@hotmail.com, tang_kaifu@yahoo.com.cn (K.-F. Tang), renhong0531@vip.sina.com (H. Ren).

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In mammals, non-coding RNAs are highly coincident with allele-specific silencing of imprinted genes and have a proven role in allele-specific gene expression [11]. The biochemistry of how non-coding RNAs regulate transcription is the subject of intense research. Mechanisms such as RNA interference may have deep phylogenetic roots. It has been reported that RNA-induced initiation of transcriptional gene silencing (RITS) complex contains *Dicer*-generated small RNAs; and it is widely believed that these small RNAs recruit RITS complex to all known heterochromatic loci [12,13].

At some imprinting domains, the ICR constitutes an insulator that prevents promoter–enhancer interactions, when unmethylated [4]. Recently, Lei and Corces reported that RNAi components influence the nuclear organization of a chromatin insulator [14]. In addition to DNA methylation and histone modifications, polycomb group proteins are also important for the somatic maintenance of imprinting [4]. Grimaud showed that RNAi components are required for nuclear clustering of Polycomb group response elements and regulate the nuclear organization of PcG chromatin targets [15]. Seitz and colleagues demonstrated that the imprinted mouse distal chromosome 12 locus encodes two miRNA genes expressed from the maternally inherited chromosome and antisense to a retrotransposon-like gene (Rtl1) expressed only from the paternal allele [16].

Repeat sequence is critical for the initiation of heterochromatin formation, and this ability is dependent on the RNA interference (RNAi) machinery. Tandem repeats have been found in close vicinity to several imprinted genes in mice and humans, for example, there are a 36 bp motif repeated 15 times in a continuous array located between 2 and 2.5 kb downstream of *PHLDA2* and a mixed 15/20 bp motif repeated ~100 times in a continuous array spanning the interval 7–9 kb upstream of the *PHLDA2* transcription start site [17]. In addition, Haussecker and Proudfoot reported that *Dicer* is required for chromatin silencing of intergenic transcription in the human beta-globin cluster [18].

Of particular interest, genomic imprinting plays a special role in placental biology and embryo development [19] and *Dicer* knockout mice show very early embryonic lethality [20]. The above observations suggest that RNAi machinery may regulate the chromatin structure and allelic expression of imprinted genes. However, Fukasawa recently reported that reduced expression of *Dicer1* did not significantly affect the maintenance and reprogramming of imprinting in mice [21]. Therefore, we speculated that *Dicer* might regulate the chromatin structure of imprinted genes. To test this hypothesis, we knocked down *Dicer* in HEK293 cells, and checked the chromatin structure and expression of imprinted genes on human chromosome 11p15.5. Our results suggest that although *Dicer* does not have function in regulation of genomic imprinting, it can regulate the chromatin structure and the expression of *PHLDA2*.

2. Materials and methods

2.1. Construction of siRNAs

siRNAs were prepared using *Silencer* siRNA Construction Kit (Ambion). Briefly, sense and antisense oligonucleotide templates were annealed with T7 promoter primer, and then filled in with Klenow DNA Polymerase. Using the filled-in dsDNAs as templates, sense and antisense RNAs were transcripted with T7 RNA polymerase. Sense and antisense RNAs were annealed and the single stranded leader sequences were removed by digesting the dsRNA with a single-strand specific ribonuclease. *Dicer* siRNA targeting sequence is 5′-AAGGCT-TACCTTCTCCAGGCT-3′; Control siRNA targeting sequence is 5′-AATTCTCC-GAACGTGTCACGT-3′, which has no homology in human genome.

2.2. Cell culture and transfection

HEK293 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). All cultures were maintained at 37 °C in a moist atmosphere containing 5% CO_2 in air. Transfections were carried out using siPORT NeoFX (Ambion, cat #4510) according to the manufacturer's instructions. Briefly, cells were trypsinized and plated in 6-well plates at 5×10^5 cells per well for 30 min before transfection. Transfection complexes were prepared in Opti-mem serum-free medium (Invitrogen) by mixing 5 μL of siPORT NeoFX Transfection reagent and 100 nM of siRNA. The cells and medium were harvested 72 h after transfection.

2.3. Growth arrest conditions

Growth arrest by serum deprivation: HEK293 cells were plated in 6-well plates at a density of 5×10^5 cells per well in RPMI 1640 medium containing 10% FBS and allowed to attach to the tissue culture plate. After 12 h, the medium was replaced with low-serum medium (containing 0.5% FBS). Cells were incubated for 3 days.

Growth arrest by contact inhibition: HEK293 cells were plated at a density of 1.2×10^6 cells per well in RPMI 1640 medium containing 10% FBS and incubated for 3 days.

2.4. Western blotting analysis

Cells were collected on day 3 posttransfection, washed in PBS and lysed in RIPA buffer. Samples were denatured at 100 °C for 5 min. Equal amounts of total protein were loaded to each well for electrophoresis in 8% SDS-polyacrylamide gels and then transferred to polyvinylidine fluoride microporous membranes (Millipore Corporation, Billerica, MA, USA). Membranes were then incubated with primary antibody followed by incubation with horseradish peroxidase-linked secondary antibodies. The primary antibodies used include anti-*Dicer* (Abcam, cat #ab1460) and anti-*GAPDH* (Kangchen, Shanghai).

2.5. Real-time RT-PCR analysis

Total RNA was prepared using TRIZOL (Life Technologies) and reverse transcribed using MMLV reverse transcriptase (TOYOBO CO, Japan) according to the manufacturer's protocol. The resulting cDNA was amplified with primer pairs in duplicate and amplification was monitored using SYBRGreen chemistry on Roche Lightcycler2.0. Oligonucleotide sequences (forward, reverse) were for *H19*: CCCACAACATGAAAGAAATGGTGC, CACCTTCGAGAGCC-GATTCC; *IGF2*: TTGCTGCTTACCGCCCCAG, GCACTCCTCAACGATGC-CAC; *PHLDA2*: TGCCCATTGCAAATAAATCACT, CTGCCCGCCCATTCCT; *GAPDH*: ATGACATCAAGAAGGTGGTG, CATACCAGGAAATGAGCTTG.; *CDKN1C*: GGACGAGACAGGCGAACC, AGAGGACAGCGAGAAGAAGG.

2.6. Northern blotting

20 μg total RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel, and transferred to a positively charged nylon membrane (Sigma). The membrane was hybridized to a probe obtained by labeling the *PHLDA2* or *GAPDH* gene with [α-^{32}P] dCTP, using a random prime DNA labeling kit (Promega). The primers for RT-PCR were used to generate probe DNAs.

For miRNA northern, 30 μg total RNA was separated by electrophoresis on a 15% PAGE gel and transferred to a positively charged nylon membrane (Sigma). The membrane was hybridized to probes obtained by labeling the oligoes with [γ-^{32}P] ATP using T4 PNK (Promega). Probe for

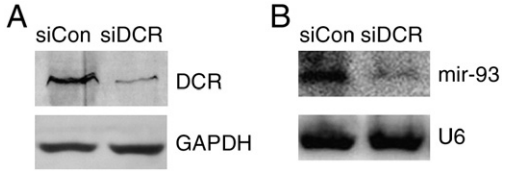

Fig. 1. Functional knockdown of *Dicer*. HEK293 cells were transfected with siDCR and siCon, and collected 3 days later. (A) Western blot showed that *Dicer* protein was reduced. (B) Northern blotting indicated that mir-93 was significantly reduced.

mir-93 was: CTACCTGCACGAACAGCACTTT; U6 probe was: AAAAATATGGAACGCTTCACGAATTTGCGTGTCATC.

2.7. Chromatin immunoprecipitations

ChIP was done by using Anti-acetyl-Histone H3 (Lys9) (upstate Biotechnology) according to the manufacturer's protocol. Immunoprecipitated DNA was quantified by real-time PCR. For *PHLDA2*, we used the same primers as for *PHLDA2* RT-PCR. Primers for *GAPDH* were: TCGGTGCGTGCCCAGTTGAACC and ATGCGGCTGACTGTCGAACAGGAG.

2.8. Bisulfite Sequencing

Bisulfite treatment of genomic DNA extracted from *Dicer* knockdown cells and control cells was carried out using EpiTect Bisulfite Kit (Qiagen, 59104) . The *PHLDA2* promoter was amplified from the bisulphite treated DNA using forward primer 5′-GGGAGATAGTYGGGATTAG-3′ and reverse primer 5′-CCCTAATCCAACAAAAATTAC-3′. The amplified product was cloned into pMD18-T simple vector (Takara); and subsequently sequenced with BigDye™ Terminator Cycle Sequencing Kit.

3. Results

3.1. Functional knockdown of Dicer with specific siRNA

To abrogate *Dicer* activity, we designed a series of siRNAs targeting different regions of *Dicer* mRNA. HEK293 cells were transfected with various siRNAs at the concentration of 100 nM and possible changes in protein levels were examined via Western blot. One siRNA, siDCR, efficiently depleted *Dicer* protein, while the *GAPDH* protein control was unchanged (Fig. 1A).

Since *Dicer* is an enzyme, small amount of *Dicer* enzyme may be enough to fulfill its function. *Dicer* is responsible for processing miRNA precursors into mature miRNAs [22]. To test whether this siRNA can functionally inhibit *Dicer* activity, we analyzed mature miRNA accumulation in *Dicer* knockdown cells. Northern blotting revealed that mir-93 was significantly decreased in *Dicer*-deficient cells (Fig. 1B).

3.2. PHLDA2 is upregulated in Dicer knockdown cells

There are several genes with previously documented functional imprinting on human chromosome 11p15.5, such as *H19*, *IGF2*, *CDKN1C* (p57KIP2), *Mash-2*, *KVLQT1* and *PHLDA2* [17]. We analyzed the expression of some of these genes in *Dicer*-deficient cells, real-time RT-PCR indicated that the expression of *H19*, *IGF2* and *CDKN1C* was not significantly changed; However, the level of *PHLDA2* was increased about 5-fold in *Dicer* knockdown cells (Fig. 2A). The upregulation of *PHLDA2* in *Dicer*-deficient cells was further confirmed by northern blotting analysis (Fig. 2B).

3.3. PHLDA2 upregulation is the result of increased H3K9 acetylation

If the upregulation of *PHLDA2* is due to loss of imprinting, we would expect that the level of upregulation should be 2-fold and that the physically and mechanistically linked imprinted gene, *CDKN1C*, should also be upregulated 2-fold in *Dicer*-deficient cells. However, our result indicated that *PHLDA2* mRNA was increased 5-fold and that *CDKN1C* mRNA level was not changed (Fig. 2A). Therefore, we speculate that the upregulation of *PHLDA2* in *Dicer*-deficient cells is due to activation of chromatin. To test this hypothesis, we analyzed acetylation state of H3K9 at *PHLDA2* locus in *Dicer*-deficient cells. Anti-acetyl-Histone H3 (Lys9) antibody precipitated DNA was quantified by real time PCR, our result demonstrated that acetylation of H3K9 at the *PHLDA2* locus was significantly increased, while the acetylation of H3K9 was not changed at *GAPDH* locus (Fig. 3A).

Whether DNA methylation is related to RNAi in mammal is controversial. Morris reported that siRNAs targeting to the promoter region of *EF1A* could induce DNA methylation [23]; however, Steer and Schultz groups reported that dsRNA could not induce DNA methylation [24,25]. To address whether upregulation of *PHLDA2* is linked to DNA methylation, bisulfite sequencing was performed. Our results showed that the DNA methylation state at the promoter region of *PHLDA2*

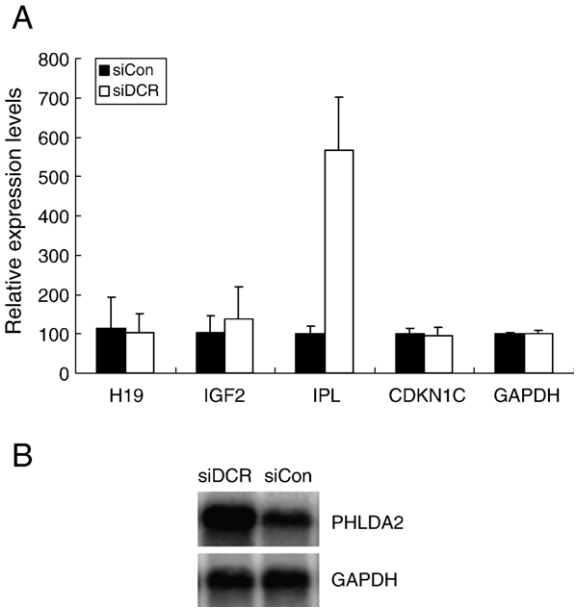

Fig. 2. *PHLDA2* was increased in *Dicer* knockdown HEK293 cells. (A) Real time RT-PCR indicated that *PHLDA2* was increased in *Dicer* knockdown cells, while the levels of *GAPDH*, *H19*, *IGF2* and *CDKN1C* were not significantly changed. The expression levels of different genes in *Dicer*-deficient cells are represented as relative values normalized to these in control cells, which are defined as 100. (B) The upregulation of *PHLDA2* was confirmed via northern blotting.

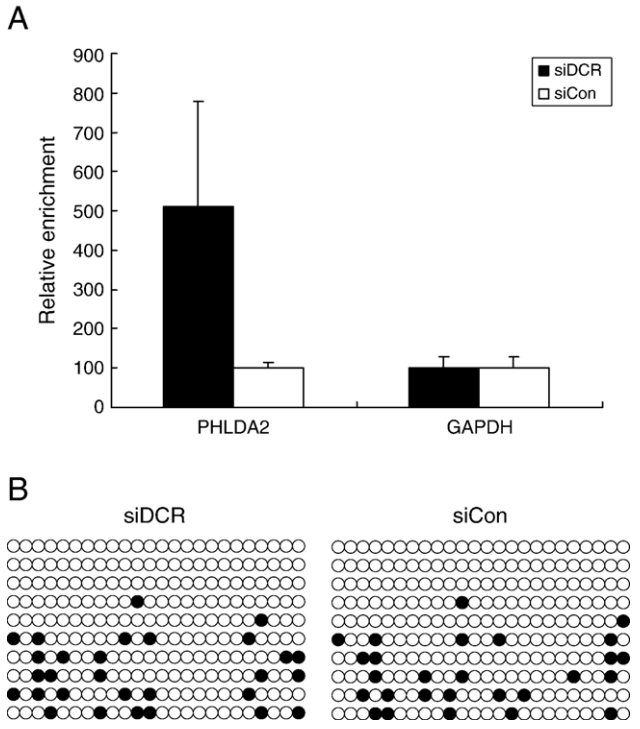

Fig. 3. *Dicer* knockdown led to increased H3K9 acetylation at *PHLDA2* locus. HEK293 cells were transfected with siCon or siDCR, and collected 3 days later. (A) H3K9 acetylation status was checked by Chromatin immunoprecipitation. Anti-acetyl-Histone H3 (Lys9) antibody precipitated DNA was quantified using real time PCR. The enrichment in *Dicer*-deficient cells is expressed as a relative value normalized to that in control cells, which is defined as 100. (B) Methylation state of *PHLDA2* promoter was not changed in *Dicer* knockdown cells. Each circle represents a single CpG dinucleotide on the strand, a methylated cytosine (●) or an unmethylated cytosine (○). For clarity, only the first 20 CpG dinucleotides from each CpG island are shown.

in *Dicer* knockdown HEK293 cells was not significantly different from that in the control cells (Fig. 3B).

3.4. *PHLDA2* upregulation is not a consequence of growth arrest

It has been demonstrated that *Dicer* is essential for cell proliferation [26]. We also found that *Dicer* knockdown caused a reduction in cell proliferation in HEK293 cells (Data not shown). *PHLDA2* is believed to be a growth-inhibitory gene [27,28]. Therefore, the upregulation of *PHLDA2* could be a consequence of growth arrest. To test this hypothesis, we checked *PHLDA2* expression in growth-arrested HEK293 cells induced by either serum deprivation or contact inhibition. Our result indicated that *PHLDA2* was downregulated in these growth-arrested HEK293 cells, while the expression of *GAPDH* was unchanged (Fig. 4). This finding suggests that *PHLDA2* upregulation might be a direct result of *Dicer* depletion rather than the consequence of growth arrest induced by *Dicer* knockdown.

4. Discussion

PHLDA2 is one of the several genes in the imprinted gene domain of 11p15.5, which is considered to be an important tumor suppressor gene region. Alterations in this region may be associated with the Beckwith–Wiedemann syndrome, Wilms tumor, rhabdomyosarcoma, adrenocortical carcinoma, and lung, ovarian, and breast cancer [17,29]. *PHLDA2*, a homologue of the mouse *TDAG51* gene, is the first apoptosis-related gene found to be imprinted. Lee and Feinberg reported that the maternal allele was expressed in normal human development, except in heart and testis of one specimen. However, two postnatal kidney samples showed biallelic expression [30]. In contrast, Qian and colleagues found *PHLDA2* imprinted only in placenta and liver [17]. A number of tumors show loss of imprinting (LOI) of genes located on human chromosome 11p15.5 [29,31,32]. Interestingly, *PHLDA2* shows retention of imprinting in most tumor specimens [33]. The mouse homologue is essential for *Fas* (*CD95*, *Apo-1*) expression and susceptibility to apoptosis in a T lymphocyte cell line [34]. *PHLDA2* encodes a small cytoplasmic protein with a pleckstrin-homology (PH) domain, which has the phosphoinositide binding capacity [35]. Frank and colleagues demonstrated that there was consistent overgrowth of placentas in the *PHLDA2* null mice [27], and overexpression of *PHLDA2* in mice led to growth inhibition [28]. These results support the hypothesis that *PHLDA2* is a growth-inhibitory gene. In this work, we demonstrated that *PHLDA2* was upregulated in *Dicer*-deficient cells and that upregulation of *PHLDA2* is not a consequence of growth arrest. Therefore, the upregulation of *PHLDA2* may be a candidate for contributing to the reduced growth rate of *Dicer*-deficient cells. We deduce that *PHLDA2* might be upregulated in *Dicer* knockout mice, and that upregulation of *PHLDA2* might inhibit the placental growth and hence lead to embryonic lethality.

These are a 36 bp motif repeated 15 times in a continuous array located between 2 and 2.5 kb downstream of *PHLDA2* and a mixed 15/20 bp motif repeated about 100 times in a continuous array spanning the interval 7–9 kb upstream of the *PHLDA2* transcription start site [17]. Transcripts derived from these repetitive sequences may facilitate heterochromatin formation and influence *PHLDA2* expression. *Dicer* knockdown may

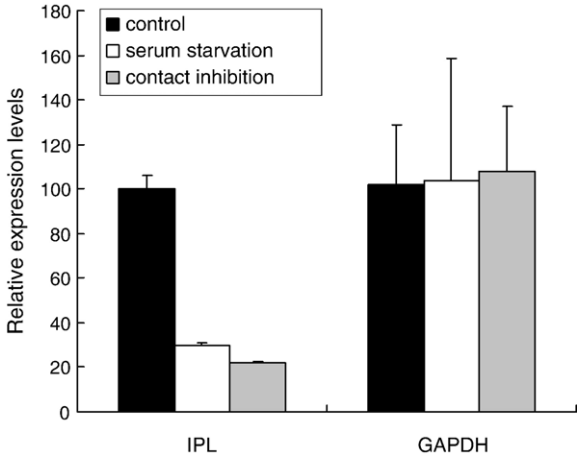

Fig. 4. *PHLDA2* expression was decreased in growth-arrested HEK293 cells. Cells were arrested by serum deprivation or by contact inhibition for 3 days, *PHLDA2* expression level was quantified by real time RT-PCR.

disrupt this heterochromatin and hence activate *PHLDA2* expression. Indeed, our results demonstrated that *Dicer* knockdown led to increased H3K9 acetylation at *PHLDA2* locus, which suggests that the upregulation of *PHLDA2* is the result of chromatin activation.

There is still another possibility that the upregulation of *PHLDA2* is a consequence of loss of imprinting. If this is the case, the level of upregulation should be 2-fold. In addition, imprinted genes are regulated as a group of genes close to each other and *CDKN1C* is physically and mechanistically linked to *PHLDA2*, therefore, *CDKN1C* should also be upregulated 2-fold if the upregulation of *PHLDA2* is the result of loss of imprinting. However, our data indicated that *PHLDA2* mRNA was increased 5-fold and *CDKN1C* mRNA was not changed in *Dicer* knockdown cells. Taken together with the report that reduced expression of *Dicer1* did not affect the maintenance and reprogramming of imprinting [21], we may rule out the possibility that upregulation of *PHLDA2* is a consequence of loss of imprinting.

It has been reported that RNAi machinery is involved in the regulation of chromatin structure and that mutants in RNAi machinery leads to a loss of heterochromatin at the centromeres [6–10]. Haussecker and proudfoot demonstrated that RNAi-dependent chromatin silencing in vertebrates is not restricted to the centromeres [18]. Notably, RNAi components have been found to be distributed throughout all heterochromatin domains [13,36]. Therefore, the chromatin structure of tandem repeats in close vicinity to *PHLDA2* might be regulated by RNAi machinery. Because tandem repeats have been found both upstream and downstream of *PHLDA2*, the chromatin activation of *PHLDA2* in *Dicer* knockdown cells may be the result of heterochromatin disruption of these tandem repeats.

Although RNAi is linked to DNA methylation in plants, whether DNA methylation is related to RNAi in mammal is controversial. Morris reported that siRNAs targeting to the promoter region of *EF1A* could induce DNA methylation [23]; However, Steer and Schultz groups reported that dsRNA could not induce DNA methylation [24,25]. Our results indicated that DNA methylation state at *PHLDA2* locus is not regulated by *Dicer*.

Given the fact that the expression of *CDKN1C*, *H19* and *IGF2* is not changed in *Dicer*-deficient cells and that genomic imprinting is not affected in *Dicer1*-hypomorphic mice, we cannot make a general conclusion that *Dicer* is able to regulate the expression of imprinted genes. However, we have indeed demonstrated that *Dicer* knockdown in HEK293 cells could activate the chromatin at *PHLDA2* locus and upregulate the expression of *PHLDA2*. The upregulation of *PHLDA2* may be a candidate for contributing to the reduced growth rate of *Dicer*-deficient cells and the very early embryonic lethality in *Dicer* knockout mice.

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