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MicroRNA-323-3p Regulates the Activity of Polycomb Repressive Complex 2 (PRC2) via Targeting the mRNA of Embryonic Ectoderm Development (*Eed*) Gene in Mouse Embryonic Stem Cells*

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Background: PRC2 is involved in many biological processes, yet how the function of PRC2 is regulated is largely unknown.
Results: The 3′-UTR of *Eed* mRNA contains miR-323-3p-binding sites. Binding of miR-323-3p to *Eed* mRNA results in reduced EED protein abundance and cellular H3K27me3 levels in mouse and human cells.
Conclusion: miR-323-3p regulates PRC2 activity via targeting *Eed*.
Significance: The work identifies miR-323-3p as a new regulator of PRC2.

PRC2 (Polycomb repressive complex 2) mediates epigenetic gene silencing by catalyzing the triple methylation of histone H3 Lys-27 (H3K27me3) to establish a repressive epigenetic state. PRC2 is involved in the regulation of many fundamental biological processes and is especially essential for embryonic stem cells. However, how the formation and function of PRC2 are regulated is largely unknown. Here, we show that a microRNA encoded by the imprinted *Dlk1-Dio3* region of mouse chromosome 12, miR-323-3p, targets *Eed* (embryonic ectoderm development) mRNA, which encodes one of the core components of PRC2, the EED protein. Binding of miR-323-3p to *Eed* mRNA resulted in reduced EED protein abundance and cellular H3K27me3 levels, indicating decreased PRC2 activity. Such regulation seems to be conserved among mammals, at least between mice and humans. We demonstrate that induced pluripotent stem cells with varied developmental abilities had different miR-323-3p as well as EED and H3K27me3 levels, indicating that miR-323-3p may be involved in the regulation of stem cell pluripotency through affecting PRC2 activity. Mouse embryonic fibroblast cells had much higher miR-323-3p expression and nearly undetectable H3K27me3 levels. These findings identify miR-323-3p as a new regulator for PRC2 and provide a new approach for regulating PRC2 activity via microRNAs.

Polycomb group proteins are key epigenetic regulators for genes involved in development and cell fate specification (1–4). Polycomb group proteins function mainly via two protein complexes, PRC1 (Polycomb repressive complex 1) and PRC2, to repress gene expression. PRC2 is mainly responsible for the dimethylation and trimethylation of histone H3 at Lys-27 (H3K27me2 and H3K27me3, respectively), resulting in transcriptional repression of the methylated genes (5–8). PRC2 is composed of the core components EZH2, EED (embryonic ectoderm development), and SUZ12, as well as other proteins (5–7, 9). EZH2 is the catalytic subunit mediating H3K27 methylation (10), whereas EED binds to the H3K27me3 sites to stimulate the methyltransferase activity of PRC2 (11). Interruption of EED binding to H3K27me3 abolishes PRC2 activity both *in vivo* and *in vitro*, resulting in disrupted development (11).

The proper function of PRC2 is crucial for embryonic stem (ES)[3] cells. ES cells lacking EZH2, EED, or SUZ12 are unable to differentiate properly (12, 13). Such an effect is partially due to the lack of repression of pluripotent genes and aberrant derepression of lineage-specific genes during the differentiation process (13). *Eed* knock-out ES cells are able to give rise to chimeras, yet both *Ezh2* and *Eed* knock-out mice display embryonic lethality soon after implantation (14–17). However, PRC2 is not necessary for the pluripotency maintenance of ES cells, as each of the key components of PRC2 can be deleted without compromising the expression levels of pluripotent markers, such as OCT4 and NANOG (13, 17).

MicroRNAs (miRNAs) are a class of ~22-nucleotide long noncoding RNAs with many essential regulatory roles in eukaryotes. They function by binding to target mRNAs via sequence complementarity and therefore repress the translation or induce the degradation of mRNAs (18, 19). In rare cases, miRNAs can also activate the expression of target mRNAs (20). miRNAs are processed from the double-stranded stem region of hairpin-shaped precursors (21, 22). After cleavage from the precursor, the double-stranded RNA duplex will be loaded into

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[3] The abbreviations used are: ES, embryonic stem; miRNA, microRNA; iPS, induced pluripotent stem; MEF, mouse embryonic fibroblast.

the RNA-induced silencing complex, in which only the mature miRNA will be retained and the pairing strand of the RNA duplex will be degraded. For some miRNA precursors, either strand of the RNA duplex can be retained as mature miRNA in the RNA-induced silencing complex; therefore, the mature miRNAs are denoted with a 5p or 3p suffix according to whether they are derived from the 5′- or 3′-arm of the hairpin-shaped precursor, respectively (23–25).

The imprinted *Dlk1-Dio3* region on mouse chromosome 12 contains six imprinted genes and a number of maternally expressed small nucleolar RNAs and miRNAs (26–28), but the functions of these genes and miRNAs are largely unknown. Previously, we showed that the expression of miRNA clusters located within the *Dlk1-Dio3* region correlates with the developmental potential of mouse ES cells and induced pluripotent stem (iPS) cells (29). The expression of miRNAs within the *Dlk1-Dio3* region in mice is much higher in iPS cells with the ability to develop normally (4n-iPS cells) via the tetraploid complementation assay compared with iPS cells without this independent developmental ability (2n-iPS cells) (29). However, how this expression difference affects the developmental ability of ES cells is still obscure.

Here, we show that miR-323-3p, an miRNA from the *Dlk1-Dio3* region, targeted mRNAs encoding the EED protein of PRC2. Such a target relationship seems to be conserved among mammals. The pairing between miR-323-3p and *Eed* mRNA interrupted the methyltransferase activity of PRC2 and resulted in reduced H3K27me3 levels in high miR-323-3p-expressing cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—Six previously reported iPS cell lines were used in this study (30). Two ES cell lines (ESC2 and CL11) were also included. All pluripotent stem cells were cultured in DMEM supplemented with 15% FBS, 0.1 mM β-mercaptoethanol, 1000 units/ml leukemia inhibitory factor, and 0.1 mM nonessential amino acids. Mouse embryonic fibroblasts (MEFs), L-02, BEL-7402, and HepG2 cells were cultured in DMEM supplemented with 10% FBS.

miRNA Target Prediction and Luciferase Reporter Assay—Putative miRNA-binding sites on the 3′-UTR of *Eed* mRNA were identified by TargetScan. Reporter vectors were created by cloning the wild-type or mutant 3′-UTR of *Eed* mRNA (starting from the stop codon until the end of sequence NM_021876) into the pMIR-REPORT miRNA expression reporter control vector (Applied Biosystems). Firefly and *Renilla* luciferase activities were analyzed using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol. The primer pair for miR-323-3p-binding sites within the 3′-UTR of *Eed* was 5′-CGAGCTCAGTCTGTTGTCTGTAAAATAGAA-3′ (forward) and 5′-GAAGCTTGTAAACATTACAAAAAGATATGGT-3′ (reverse).

RNA Extraction and Real-time PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Afterward, 1 μg of RNA from each sample was extracted and reverse-transcribed into cDNA using a random primer and subjected to real-time PCR. Real-time quantification was performed using a Stratagene Mx3000P quantitative PCR system (Genetimes Technology). The reactions were incubated in a 96-well plate at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All reactions were run in triplicate. The primer pairs used were as follows: *Eed*, 5′-ATGCTGTCAGTATTGAGAGTGGC-3′ (forward) and 5′-GAGGCTGTTCACACATTTGAAAG-3′ (reverse); *Suz12*, 5′-AGCTCTGCCACAGCAGGTTCA-3′ (forward) and 5′-TGCTTTTGTTCTTTTTGGCCTGCAA-3′ (reverse); *Ezh2*, 5′-CGCGGGACTAGGGAGTGTTCAGT-3′ (forward) and 5′-AGTACATTATAGGCACCGAGGCGA-3′ (reverse); and *Gapdh*, 5′-TCCCACTCTTCCACCTTCGATGC-3′ (forward) and 5′-GGGTCTGGGATGGAAATTGTGAGG-3′ (reverse). The primer pairs for developmental regulatory genes were as described in a previous report (2). For miR-323-3p, total RNA was isolated as described above. Reverse transcription of 1 μg of RNA into cDNA was carried out using an All-in-One™ miRNA first-strand cDNA synthesis kit (Genecopoeia). Subsequent real-time PCR, using miR-323-3p-specific primers and universal adaptor PCR primers (Genecopoeia), was performed with the Stratagene Mx3000P quantitative PCR system.

Transfection of miRNA Mimics and Inhibitor—Synthesized miR-323-3p mimics were purchased from Ambion (catalog no. MSY0000551), and the synthesized miR-323-3p inhibitor was purchased from Genecopoeia (catalog no. MmiR-AN0410-SN-5). About 30–50% confluent cells were transfected with 50 nM miR-323-3p mimics, mimic scrambled control, or miR-323-3p inhibitor and inhibitor scrambled control using Lipofectamine RNAiMAX (Invitrogen). RNA and proteins were harvested 48 h after transfection.

Western Blot Analysis—Western blotting was performed as described previously (31). The commercial antibodies used were anti-EED (Santa Cruz Biotechnology catalogue no. 133537), anti-EZH2 (BD Biosciences catalogue no. 612667), anti-SUZ12 (Abcam catalogue no. 12073), and anti-H3K27me3 (Millipore catalogue no. 07-449).

Chromatin Immunoprecipitation—MEFs were grown in MEF medium, and iPS and ES cells were maintained in ES cell medium. Approximately 1×10^7 cells were used for each ChIP assay. ChIP assays were performed according to a previous protocol (32). The chromatin DNA precipitated by either normal rabbit IgG (control) or anti-H3K27me3 polyclonal antibodies was purified with a Qiagen PCR purification kit. The samples were analyzed by real-time PCR. The primer pair for the H3K27me3-binding site was 5′-CGGAGTTGTGCCAAGGTGCACT-3′ (forward) and 5′-ATCTGTGCGCAAGGCACGGT-3′ (reverse).

RESULTS

miR-323-3p Targets the Eed 3′-UTR in Mouse ES Cells—Using bioinformatic analysis, we identified two putative binding sites of miR-323-3p within the 3′-UTR of mouse *Eed* mRNA (Fig. 1*A*). The pairing between miR-323-3p and the first binding site in the *Eed* mRNA 3′-UTR is conserved among dogs, pigs, rats, mice, and humans (Fig. 1*A*). To validate the authentication of the prediction results, we constructed luciferase expression plasmids containing the *Eed* 3′-UTR segment with the putative miR-323-3p-binding sites. Cotransfection of the miR-323-3p

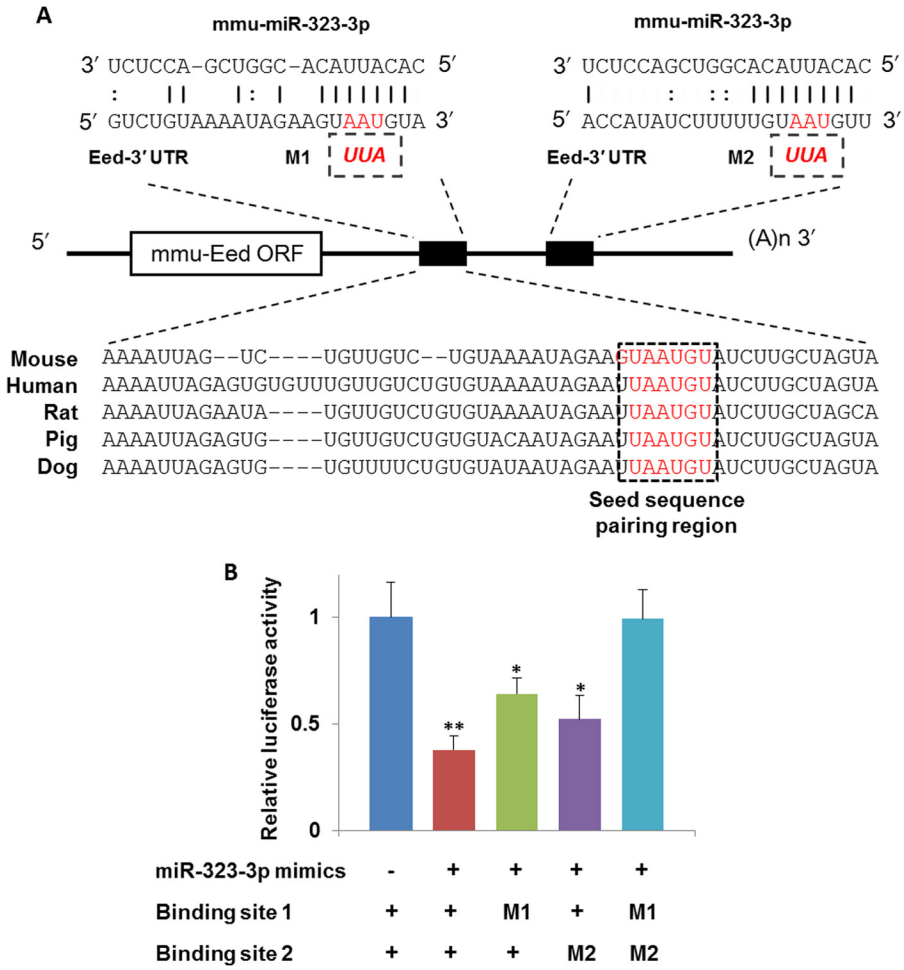

FIGURE 1. **Luciferase reporter assays demonstrating the target relationship between miR-323-3p and *Eed* mRNA.** *A*, the two predicted miRNA-binding sites within the 3′-UTR of *Eed* mRNA and their conservation analysis. The pairing between miR-323-3p and the two putative binding sites on the 3′-UTR of *Eed* mRNA is shown. Nucleotides selected for mutagenesis are shown in *red*, and the mutated nucleotides are shown in *dashed boxes* under the alignments. The pairing between miR-323-3p and the first binding site on the *Eed* mRNA 3′-UTR is conserved among dogs, pigs, rats, mice, and humans, whereas the second miR-323-3p target site is not conserved. Conserved nucleotide pairing with the seed region of miR-323-3p is shown in *red* in the alignment of *Eed* 3′-UTRs among species. *B*, luciferase activity assays detecting the authentication of miR-323-3p-binding sites on the 3′-UTR of *Eed*. Cotransfection of control miRNA and luciferase reporters with the wild-type 3′-UTR was used as a control. In other groups, miR-323-3p mimics were cotransfected with luciferase reporters containing wild-type 3′-UTR or the 3′-UTR with mutated binding site 1, with mutated binding site 2, and with both mutations. *, $p < 0.05$; **, $p < 0.001$ (Student's *t* test, n = four independent experiments).

mimics and the constructed *Eed* 3′-UTR expression plasmids into mouse ES cells (ESC2 line) significantly suppressed *Eed*-luciferase activity (Fig. 1*B*). Mutation of either of the two binding sites did not abolish the inhibitory effect of miR-323-3p, although the expression level of the luciferase reporter increased slightly. Only mutation of both miR-323-3p-binding sites completely eliminated the inhibitory effects (Fig. 1*B*). These data demonstrate that *Eed* is indeed the direct target of miR-323-3p.

iPS Cells with Full Developmental Ability Have Higher miR-323-3p Expression as Well as Reduced Eed and H3K27me3 Levels—We conducted real-time PCR to detect the abundance of miR-323-3p in ES (CL11 and ESC2), 4n-iPS (IP14D-1, IP14D-6, IP16DT-2A, and IP14DN-5), and 2n-iPS (IP20D-3 and IP36D-3) cells. The expression of miR-323-3p was much higher in ES and 4n-iPS cells than in 2n-iPS cells (Fig. 2*A*). Consistent with the above proven targeting relationship, the abundance of both *Eed* mRNA and EED protein was much lower in ES and 4n-iPS cells than in 2n-iPS cells (Fig. 2, *B* and *C*). Used as controls, the expression levels of other components of PRC2, SUZ12 and EZH2, did not vary in ES, 4n-iPS, and 2n-iPS cells (Fig. 2, *B* and *C*). In concert with the decreased EED abundance, the H3K27me3 level was also reduced in 4n-iPS and ES cells, as evidenced by a Western blot experiment using anti-H3K27me3 antibody and the ChIP PCR experiment (Fig. 2, *C* and *D*).

To test whether the positive correlation between miR-323-3p expression and pluripotency as evidenced by results obtained with the 2n-iPS and 4n-iPS cells also exists in other types of stem cells, we examined the expression of miR-323-3p and *Eed* in ES and neural stem cells. The less pluripotent neural stem cells had lower endogenous miR-323-3p expression and higher *Eed* mRNA levels than ES cells (Fig. 2*E*).

miR-323-3p Represses Eed Expression and Reduces H3K27me3 Levels in Mouse ES Cells—To examine whether miR-323-3p can indeed target *Eed* mRNA in mouse ES cells, we transiently transfected miR-323-3p into ESC2 mouse ES cells. Endogenous *Eed* mRNA and EED protein levels were measured

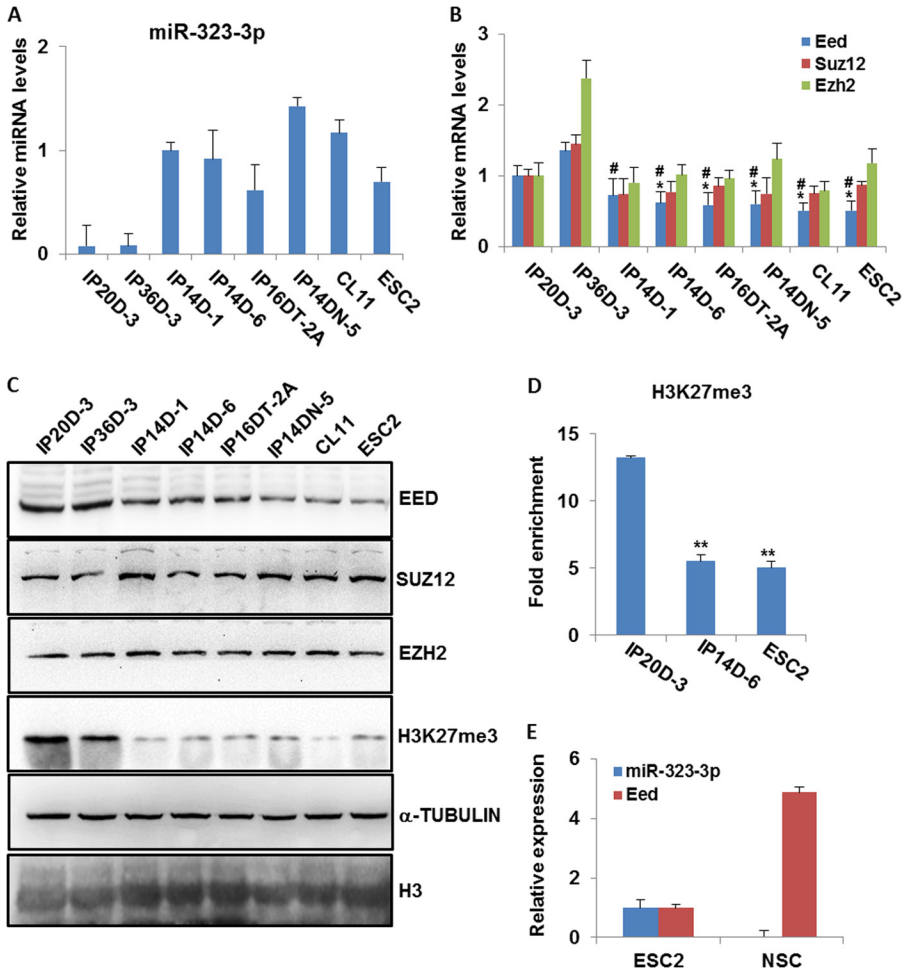

FIGURE 2. **Differential expression of miR-323-3p and *Eed* in ES and iPS cells with different developmental abilities.** *A*, expression of miR-323-3p in ES cells (CL11 and ESC2), 4n-iPS cells (IP14D-1, IP14D-6, IP16DT-2A, and IP14DN-5) with full developmental abilities, and 2n-iPS cells (IP20D-3 and IP36D-3) with incomplete developmental abilities. *B*, mRNA abundance of *Eed* and other PRC2 components (SUZ12 and EZH2) in ES, 4n-iPS, and 2n-iPS cells. *, $p < 0.05$, compared with IP20D-3; #, $p < 0.05$, compared with IP36D-3 (Student's *t* test, n = three independent experiments). *C*, protein abundance of EED, SUZ12, and EZH2 as well as the H3K27me3 levels in ES, 4n-iPS, and 2n-iPS cells. α-Tubulin and histone H3 were used as loading controls. *D*, quantitative ChIP assays detecting H3K27me3 in the *Dlk1-Dio3* region. **, $p < 0.001$ (Student's *t* test, n = three independent experiments). *E*, expression of miR-323-3p and *Eed* mRNA in ES and neural stem cells (*NSC*) detected by real-time PCR.

48 h after transfection. The expression of both *Eed* mRNA and EED protein decreased in the presence of miR-323-3p (Fig. 3, *A*, *B*, and *D*). In contrast, when the mouse ES cells were transfected with the miR-323-3p inhibitor, both the *Eed* mRNA and EED protein levels increased (Fig. 3, *A*, *B*, and *E*). Other components of PRC2 without miR-323-3p-binding sites, such as SUZ12 and EZH2, had no significant change in expression (Fig. 3, *A* and *B*). In concert with the change in EED protein abundance, the H3K27me3 level in mouse ES cells decreased upon miR-323-3p overexpression and increased when the expression of miR-323-3p was inhibited (Fig. 3, *B* and *C*).

Previous work has shown that *Eed* mutant ES cells express higher levels of developmental regulatory genes compared with wild-type ES cells (2). To further confirm the regulatory relationship between miR-323-3p and *Eed*, we detected the expression of 14 known developmental regulatory genes in miR-323-3p-overexpressing ESC2 cells; eight tested genes showed increased expression in miR-323-3p-overexpressing ESC2 cells compared with control ES cells, which was in agreement with the difference in EED levels in these cells (Fig. 3*F*). Previous studies have shown that these developmental regulatory genes are direct targets of PRC2 (2). The increased expression of these genes upon miR-323-3p overexpression indicates that miR-323-3p can regulate the function of PRC2 through modulating *Eed* expression.

miR-323-3p Is Differentially Expressed in MEFs and ES Cells and Results in Altered H3K27me3 Levels—Compared with mouse ES cells, the MEFs had much higher endogenous miR-323-3p expression (Fig. 4*A*). Consequently, the mRNA level of *Eed* was much lower in MEF cells compared with ES cells (Fig. 4*A*). Consistent with the low mRNA abundance, the expression of EED protein was barely detected in MEF cells by Western blot hybridization, whereas the other PRC2 component, EZH2, had a similar abundance in MEF and ES cells (Fig. 4*B*). We further examined the H3K27me3 level in these two types of cells; in concert with the reduced expression of EED, the H3K27me3 level was significantly reduced in MEF cells (Fig. 4*B*).

miR-323-3p Regulates Eed Expression in Human Cells—To examine whether the regulatory relationship between miR-323-3p and *Eed* mRNA is also conserved in humans, we detected the expression of miR-323-3p and *Eed* in human can-

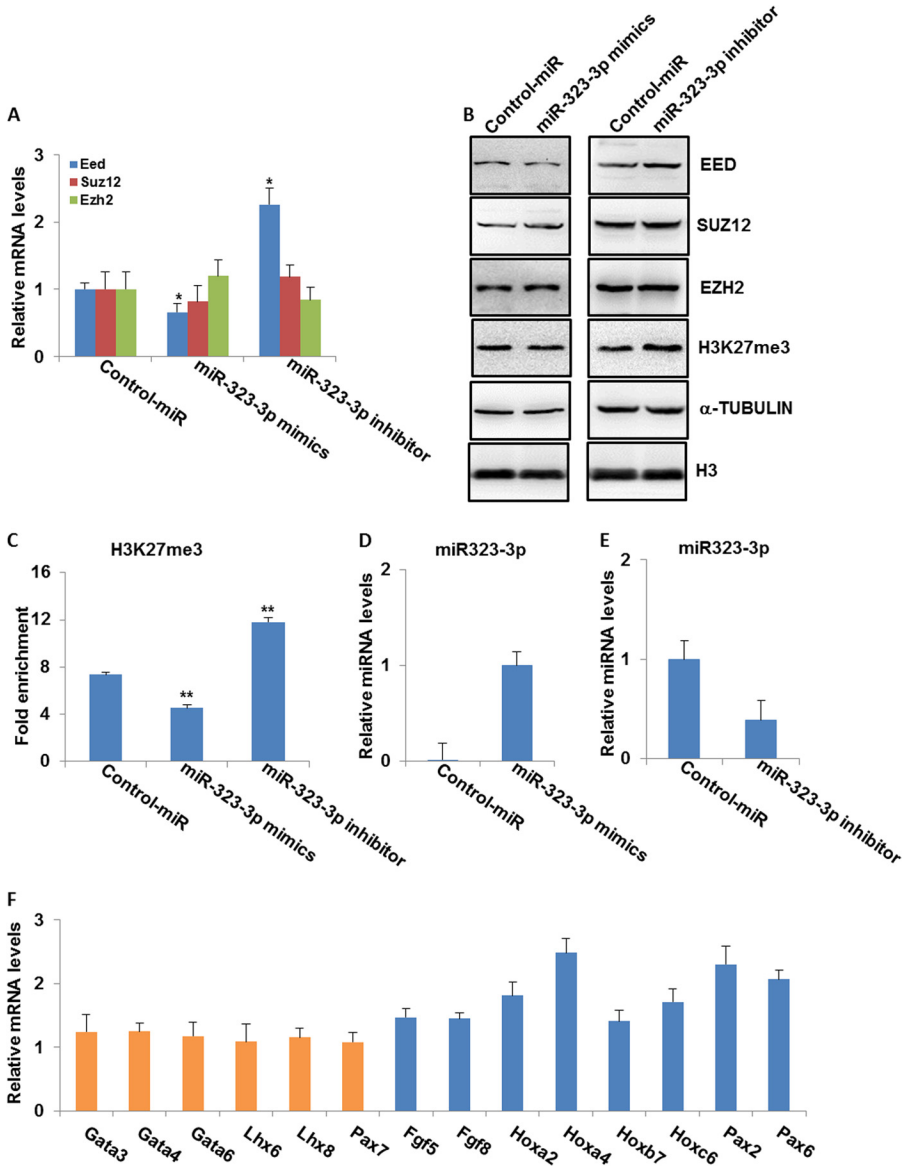

FIGURE 3. **miR-323-3p represses H3K27me3 through reduced *Eed* expression in ES cells.** *A*, real-time PCR was used to determine the mRNA abundance of *Eed*, *Suz12*, and *Ezh2* in ESC2 ES cells transfected with control miRNA (*miR*), miR-323-3p mimics, and the miR-323-3p inhibitor, respectively. *B*, Western blotting was performed to determine the abundance of EED, SUZ12, and EZH2 proteins as well as H3K27me3 levels in ESC2 ES cells transfected with control miRNA, miR-323-3p mimics, and the miR-323-3p inhibitor. *C*, quantitative ChIP assays were performed to determine the level of H3K27me3 in the *Dlk1-Dio3* region in ESC2 ES cells transfected with control miRNA, miR-323-3p mimics, and the miR-323-3p inhibitor. *, $p < 0.05$; **, $p < 0.001$ (Student's *t* test, n = three independent experiments). *D*, real-time PCR was carried out to determined miR-323-3p abundance in ESC2 ES cells overexpressing miR-323-3p mimics. *E*, real-time PCR was performed to determine miR-323-3p abundance in ESC2 ES cells overexpressing the miR-323-3p inhibitor. *F*, real-time PCR was carried out to determine mRNA abundance of developmental regulatory genes in ESC2 ES cells transfected with control miRNA and miR-323-3p mimics. The *y axis* represents the relative mRNA abundance of miR-323-3p-overexpressing cells *versus* control miRNA-overexpressing cells. Genes with a >1.4-fold increase in expression in miR-323-3p-overexpressing cells are shown in *blue*; other genes are shown in *orange*.

cer cells. Both the HepG2 and BEL-7402 liver cancer cell lines had higher miR-323-3p expression and lower *Eed* mRNA levels compared with the L-02 normal liver cell line (Fig. 5*A*). We further examined *Eed* expression upon overexpression of the miR-323-3p mimics or the miR-323-3p inhibitor in the human cancer cell line BEL-7402. The results showed that expression of *Eed* mRNA was reduced by the miR-323-3p mimics and increased by the miR-323-3p inhibitor (Fig. 5*B*).

DISCUSSION

It has been shown that PRC2 is involved in various biological processes and is especially important for the regulation of stem cell pluripotency and differentiation (33). Although the functional studies of PRC2 and its components have attracted a lot of attention, the regulatory mechanisms of PRC2 itself are still largely unknown. Here, we have shown that one of the key components of PRC2, EED, is regulated by an miRNA, miR-323-3p.

miRNAs are a class of ~22-nucleotide long noncoding RNA molecules that modulate gene expression at the post-transcriptional level by sequence complementarity (18, 19). Our results demonstrated that the binding of miR-323-3p to the 3′-UTR of *Eed* mRNA can reduce the expression of both *Eed* mRNA and EED protein. The pairing pattern between miR-

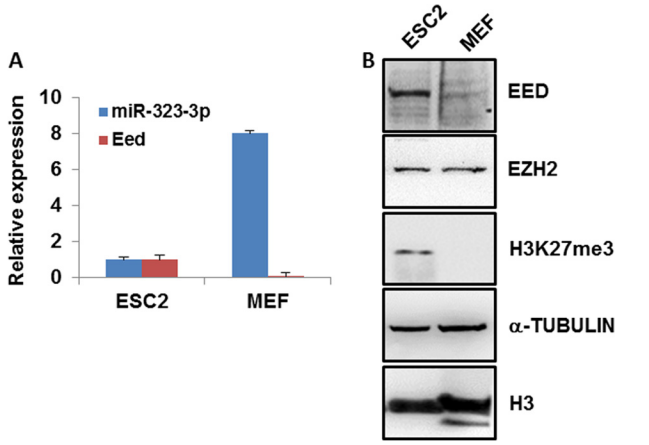

FIGURE 4. **Differential expression of miR-323-3p in MEF and ES cells.** *A*, real-time PCR was performed to determine miR-323-3p and *Eed* mRNA abundance in ES and MEF cells. *B*, abundance of EED and EZH2 proteins and H3K27me3 in ES and MEF cells. α-Tubulin and histone H3 were used as loading controls.

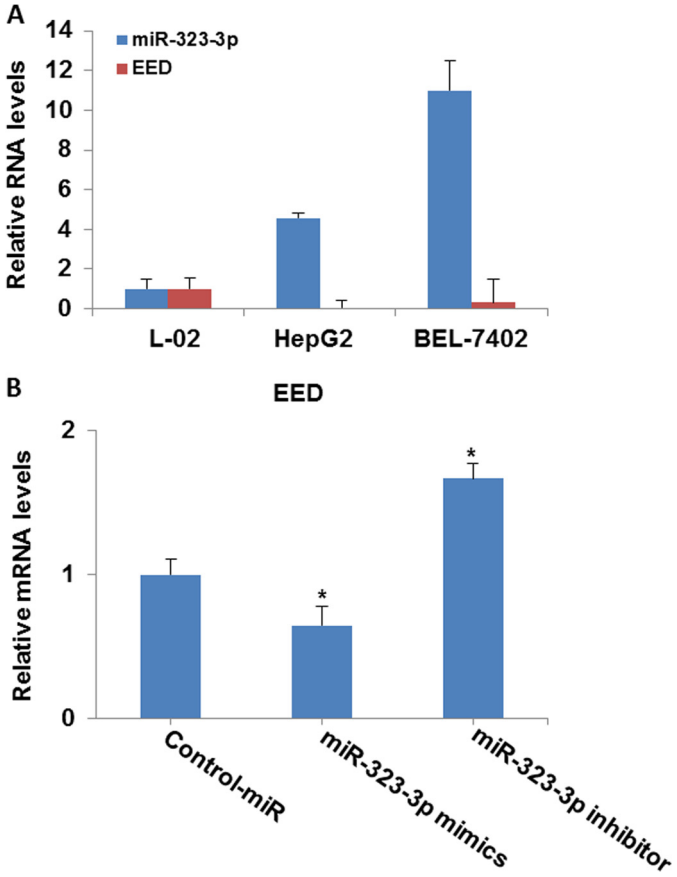

FIGURE 5. **miR-323-3p reduces *Eed* expression in human cells.** *A*, expression of miR-323-3p and *Eed* mRNA in the human HepG2 and BEL-7402 liver cancer cell lines as well as in the L-02 normal liver cell line. *B*, expression of *Eed* mRNA in the control human liver cancer cell line and in human liver cancer cell lines overexpressing miR-323-3p mimics and the miR-323-3p inhibitor.

323-3p and *Eed* mRNA seems to be conserved among mammals.

miR-323-3p belongs to an miRNA cluster located in the imprinted *Dlk1-Dio3* region on mouse chromosome 12. The miRNA cluster also contains >30 other miRNAs, including miR-299, miR-329, miR-411, among others. There is another miRNA cluster with ~10 miRNAs located next to the miR-323-3p-containing cluster within the *Dlk1-Dio3* region. Our previous study has shown that the expression of the miRNA clusters within the *Dlk1-Dio3* region positively correlates with the developmental potential of mouse ES and iPS cells (29). In ES and iPS cells with complete developmental ability, the expression of these miRNAs was higher than that in ES and iPS cells with impaired developmental ability. In concert with the target regulatory relationship, we have shown here that the expression of *Eed* mRNA and EED protein was reversely correlated with the expression level of miR-323-3p in iPS cells with different developmental abilities.

PRC2 has histone methyltransferase activity and primarily functions to trimethylate histone H3 at Lys-27. It has been shown that EED protein can mediate binding of PRC2 to H3K27me3 to reinforce the enzymatic activity of PRC2 via such a positive feedback loop (11). Thus, repression of *Eed* expression by miR-323-3p could impede the function of PRC2 by two approaches. The first is to reduce the abundance of EED protein, therefore preventing the formation of the complete PRC2 complex. The second is to prevent the reinforced enzymatic activity of PRC2 by abolishing the binding of EED to H3K27me3. Here, we have proven that reduced expression of EED caused by overexpression of miR-323-3p indeed led to decreased H3K27me3 levels in ES cells, demonstrating that miR-323-3p can interfere with the methyltransferase function of PRC2. Further experiments are need to investigate whether both of the above mentioned approaches could contribute to such an effect.

The key pluripotent regulators STAT3 and Oct4 have been reported as upstream regulators of *Eed* in ES cells (34). As the miR-323-3p-containing *Dlk1-Dio3* region has been shown to be a target of STAT3 and Oct4 (35), it is possible that the regulation of STAT3 and Oct4 on *Eed* is partially mediated through miR-323-3p.

In this study, we also found that the differentiated MEF cells had much higher miR-323-3p expression and much lower EED abundance as well as H3K27me3 levels compared with the mouse ES cells. Such results seem to be in conflict with the finding that the expression level of miR-323-3p was positively correlated with the pluripotency of iPS and ES cells. However, as there have been lines of evidence showing that inhibition of PRC2 function is required for the proper differentiation of certain types of cells, such as myoblasts and basal cells (36, 37), it is possible that in MEF cells, the function of PRC2 also could be blocked by miR-323-3p.

In summary, we have identified miR-323-3p as a regulator for the expression of *Eed* and the function of PRC2. Such regulation is conserved between mice and humans. These findings not only contribute to the regulatory network of stem cell pluripotency but also provide a new approach for modulating PRC2 functions.

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