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EED regulates epithelial–mesenchymal transition of cancer cells induced by TGF-β

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ABSTRACT

Histone methylation is involved in various biological and pathological processes including cancer development. In this study, we found that EED, a component of Polycomb repressive complex-2 (PRC2) that catalyzes methylation of lysine 27 of histone H3 (H3K27), was involved in epithelial–mesenchymal transition (EMT) of cancer cells induced by Transforming Growth Factor-beta (TGF-β). The expression of *EED* was increased during TGF-β-induced EMT and knockdown of *EED* inhibited TGF-β-induced morphological conversion of the cells associated with EMT. *EED* knockdown antagonized TGF-β-dependent expression changes of EMT-related genes such as *CDH1*, *ZEB1*, *ZEB2* and *microRNA-200* (*miR-200*) family. Chromatin immunoprecipitation assays showed that EED was implicated in TGF-β-induced transcriptional repression of *CDH1* and *miR-200* family genes through the regulation of histone H3 methylation and EZH2 occupancies on their regulatory regions. Our study demonstrated a novel role of EED, which regulates PRC2 activity and histone methylation during TGF-β-induced EMT of cancer cells.

1. Introduction

Lysine (K) methylation on the N-terminal tail of histone H3 (K4, K9, K27 and K36) plays critical roles in transcriptional regulation and maintenance of genome integrity [1,2]. Methylation of H3K4 has been associated with active transcription whereas methylation of H3K9 and H3K27 are repressive marks of chromatin. Methylation is regulated by two classes of enzymes: histone lysine methyltransferases (KMTs) and lysine demethylases (KDMs). Recent studies have revealed that the deregulation of these enzymes may contribute to the developmental defects and the pathogenesis of human diseases including cancer [1–3].

Using retroviral insertional mutagenesis in mice, we have identified hundreds of candidate cancer genes including many genes encoding KMTs and KDMs [4,5]. Previously, we reported that KDM5B, an H3K4 demethylase, down-regulated the expression of *KAT5* and *CD82* genes to increase cell invasion [6] and repressed the expression of *microRNA-200* (*miR-200*) family, thereby promoting epithelial–mesenchymal transition (EMT) of cancer cells [7]. Thus our studies indicated that histone methyl-modifying enzymes were involved not only in tumor initiation but also in tumor progression.

Tumor progression has been associated with the activation of the EMT program that is induced by extrinsic signals such as Transforming Growth Factor-beta (TGF-β) [8,9]. EMT is characterized by the changes in epithelial and mesenchymal gene expression. Especially, the down-regulation of E-cadherin is essential for EMT. Many studies on the mechanisms for E-cadherin repression demonstrated that several transcriptional repressors such as ZEB1 and ZEB2 are involved in the complex network that control EMT [10]. The plasticity of EMT suggests that epigenetic regulation such as DNA methylation, histone modification and microRNA may be involved in EMT program [11]. There are several papers including our study demonstrating the connection between E-cadherin repression and the function of histone methyl-modifying enzymes [7,12,13]. However, the role of histone methylation in EMT is just beginning to be disclosed.

Polycomb repressive complex-2 (PRC2) is known to regulate important gene expression patterns during development [14]. PRC2 is composed of essential core subunits, EZH2, SUZ12, EED, RBBP4 and RBBP7. The conserved SET domain of EZH2 contains

Abbreviations: EMT, epithelial–mesenchymal transition; TGF-β, Transforming Growth Factor-beta; miR-200, microRNA-200; ChIP, chromatin immunoprecipitation; PRC2, Polycomb repressive complex-2; H3K27me3, histone H3 trimethylated Lys27; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IgG, immunoglobulin G; shRNA, small hairpin RNA.

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the active site for catalysis of histone H3K27 methylation, a mark correlated with gene repression. Deregulation of PRC2 enzyme activity is thought to contribute to a number of human diseases including cancer. It was reported that EZH2 was overexpressed in metastatic cancer, and the expression levels were associated with tumor progression [15,16]. Overexpression of *EZH2* in immortalized mammary epithelial cells was shown to promote cell invasion through transcriptional repression of *CDH1*/*E-cadherin* gene [12], suggesting that EZH2 might drive malignant progression through an EMT program. However, the role of PRC2 core components during cancer progression including EMT remains unknown.

In this study we investigated the function of EED, one of the PRC2 core components, during TGF-β-induced EMT of cancer cells. We found that TGF-β-dependent expression changes of EMT-related genes were inhibited by *EED* knockdown and enhanced by *EED* overexpression. Mechanistic investigations suggested that EED was involved in TGF-β-induced transcriptional repression of *CDH1* and *miR-200* family genes through the modulation of EZH2 recruitment and histone H3 methylation on the chromatin.

2. Materials and methods

2.1. Plasmids and cell culture

The small hairpin RNA (shRNA)-expressing retrovirus vectors were constructed as described previously [6]. The sequences of the oligonucleotides are listed in Supplementary Table S1. We confirmed that *EED* transcripts were down-regulated with the infection of both *EED* shRNA-expressing retroviruses even in the presence of TGF-β by quantitative RT-PCR (QRT-PCR) (Supplementary Fig. S1). We also confirmed that both *EED* shRNAs caused the same effects in our EMT studies (Supplementary Fig. S2), and thus we presented the data of *EED* shRNA#1 as a representative result. Mouse *EED* cDNA was tagged with FLAG-6xHis-tag, and then cloned into pDON-5 Neo plasmid (Takara) to produce retroviruses expressing EED.

A549 human lung cancer cell line and HT29 colon cancer cell line were maintained as described previously [7]. For EMT induction, the cells were treated with 1–5 ng/ml of TGF-β (R&D Systems) for 24–72 h. The production and the infection procedures of shRNA or cDNA-expressing retroviruses were described previously [6].

2.2. Quantitative PCR

RNA preparation and QRT-PCR analysis were performed as described previously [6]. PCR data were normalized with respect to control human *GAPDH* expression. The averages from at least three independent experiments are shown with the standard deviations. *P*-values were calculated between control and the samples using Student's *t*-test. Primers used for the QPCR were described previously [6,7] and listed in Supplementary Table S1. The primers for human *EED* could also detect mouse *EED* transcripts.

For microRNA quantification, TaqMan MicroRNA Assays (Applied Biosystems) for *miR-200a* (#000502) and *miR-200c* (#002300) were used. All data were normalized with respect to *RNU6B* (#001093) expression.

2.3. Immunoblotting, cell staining and immuno-fluorescence assay

Immunoblotting was performed as described previously [7]. Anti-E-cadherin (#610181, BD Transduction Lab), anti-ZEB1 (#3396, Cell Signaling), anti-ZEB2 (#61096, Active Motif), anti-phosphorylated SMAD3 (ab51451, Abcam) and anti-GAPDH (6C5, Millipore) antibodies were used. To detect cell morphological changes, A549 or HT29 cells were stained with 0.4% crystal violet (Waldeck). To allow direct fluorescence of actin cytoskeleton, the cells were stained with 0.25 μM tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma). For indirect immunofluorescence, the specimens were incubated with anti-E-cadherin antibody and treated with Alexa546-conjugated anti-mouse IgG antibody (Invitrogen). Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI).

2.4. Chromatin immunoprecipitation (ChIP) assays

ChIP experiments were performed as previously described [6,17]. The cross-linked chromatins were immunoprecipitated with mouse antibody (anti-H3K27me3, anti-H3K4me3 [17], anti-EZH2 (#17-662, Millipore) and anti-FLAG (M2, #F1804, Sigma)). The enrichment of the specific amplified region was analyzed by QPCR and percentage enrichment of each histone modification over input chromatin DNA was shown. Primers used for QPCR correspond to the region a of *CDH1* gene, region b of *miR-200b*/*a*/*429* gene and region b of *miR-200c*/*141* gene, respectively, as described previously [7].

3. Results

3.1. EED is involved in TGF-β-induced EMT of cancer cells

To investigate the involvement of PRC2 enzyme in EMT, we first examined the changes in gene expression of PRC2 core members (*EZH2*, *SUZ12*, *EED*, *RBBP4 and RBBP7*) during TGF-β-induced EMT process. We used a lung cancer cell line, A549, because it shows rapid and clear morphological changes during EMT caused by TGF-β treatment [18]. A549 cells were treated without or with TGF-β, and RNAs were extracted from the cells and transcribed to cDNAs. Quantitative RT-PCR (QRT-PCR) indicated that only *EED* expression was clearly increased by TGF-β treatment and that the expression of other PRC2 components did not show any significant changes (Fig. 1A).

To elucidate the function of EED in EMT, we examined whether knockdown of *EED* would influence the EMT process induced by TGF-β. A549 cells were infected with the control retrovirus or the retrovirus expressing *EED* shRNA, and the infected cells were treated with or without TGF-β. After TGF-β treatment, the control cells were dispersed, elongated and assumed a fibroblast-like appearance associated with EMT (Fig. 1B). *EED* knockdown itself did not change cell shapes significantly, but inhibited morphological changes of the cells induced by TGF-β (Fig. 1B), suggesting that *EED* knockdown might antagonize TGF-β-induced EMT. To confirm this, we performed immunofluorescence assay for A549 cells using an antibody against E-cadherin, an epithelial cell marker. Untreated control A549 cells showed heterogeneous E-cadherin staining, and this staining was almost lost in TGF-β-treated cells (Fig. 1C) as described previously [18]. *EED* knockdown induced slightly stronger staining of E-cadherin on the cell membrane compared to control cells, and this staining was similarly detected after TGF-β treatment (Fig. 1C). These results suggested that the epithelial properties might be strengthened by *EED* knockdown and maintained even after TGF-β treatment. We also confirmed that actin reorganization, which was induced during TGF-β-induced EMT, was not observed in *EED* knockdown cells even after TGF-β treatment (Supplementary Fig. S3). We next examined whether *EED* knockdown would cause similar effects on another EMT model. We used a human colon cancer cell line, HT29, because it responds to TGF-β for EMT [7]. QRT-PCR revealed that *EED* expression was also induced by TGF-β in HT29 cells (Supplementary Fig. S4). TGF-β treatment induced morphological changes, disappearance of E-cadherin staining and formation of actin stress fiber

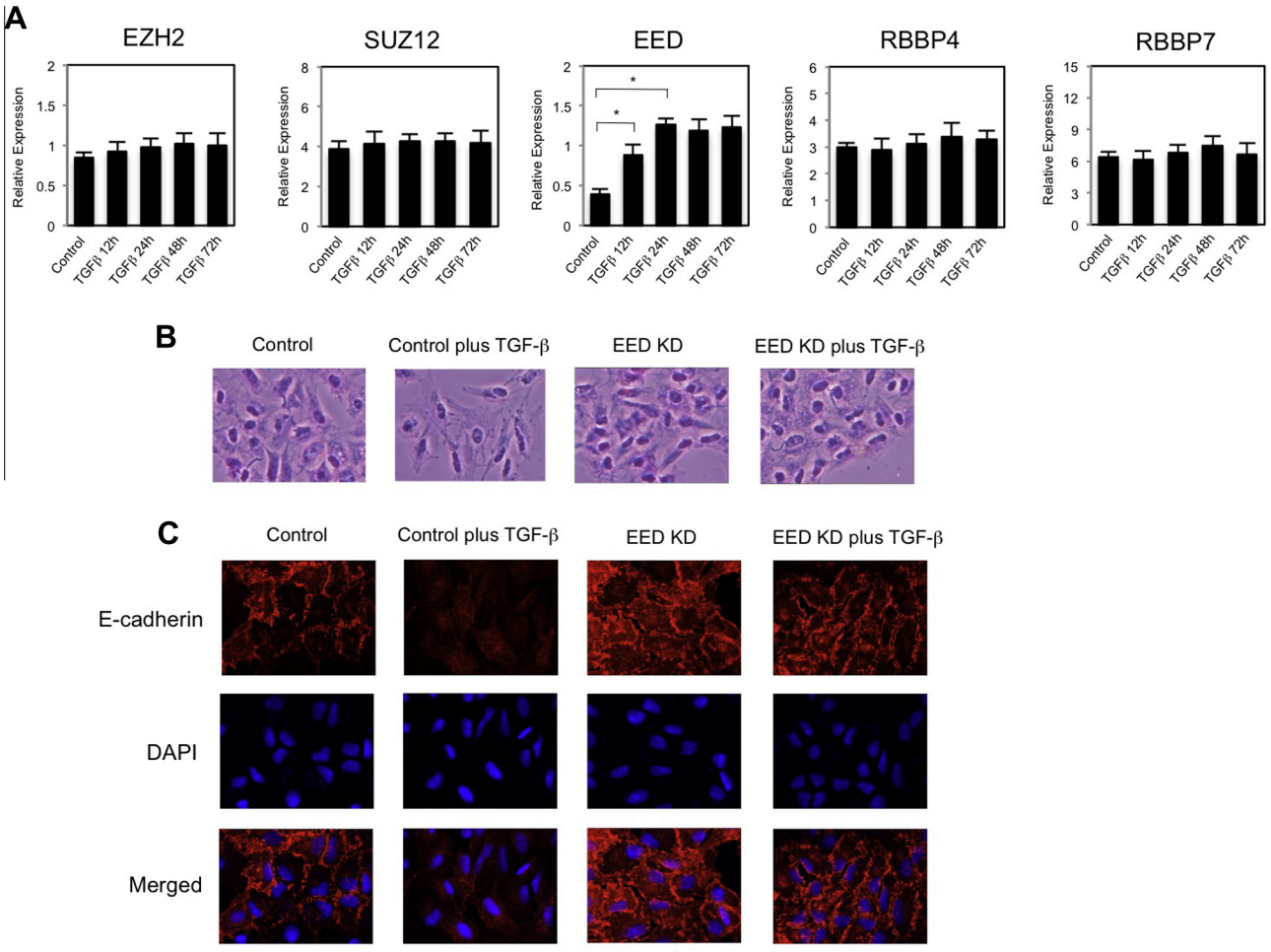

Fig. 1. (A) The expression of *EED* gene was increased during TGF-β-induced EMT of A549 lung cancer cells. QRT-PCR was performed to detect the expression of PRC2 core components in A549 cells before and after TGF-β treatment (12, 24, 48 and 72 h) (*$P < 0.01$ comparing to control). (B and C) Knockdown of *EED* antagonized TGF-β-induced morphological changes of A549 cells. A549 cells were infected with retroviruses expressing control shRNA or *EED* shRNA (*EED* sh1) without or with treatment of TGF-β for 48 h. The cells were stained with crystal violet (B) or stained with anti-E-cadherin antibody and DAPI (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in HT29 cells (Supplementary Fig. S5). However, *EED* knockdown could antagonize these TGF-β-induced phenotypes (Supplementary Fig. S5). Altogether, these results indicated that knockdown of *EED* counteracted TGF-β-induced morphological changes and cytoskeletal rearrangements of the cells characteristic of EMT.

3.2. Knockdown of EED affected the changes in expression of EMT-related genes induced by TGF-β

EMT is characterized by the changes in epithelial and mesenchymal marker gene expression [8]. Thus we analyzed the expression of an epithelial marker, *CDH1*/*E-cadherin*, and a mesenchymal marker, *FN1*/*Fibronectin*, in the *EED* knockdown cells. QRT-PCR showed that TGF-β decreased the expression of *CDH1*/*E-cadherin* mRNA in A549 cells (Fig. 2A) as previously reported [18]. *EED* knockdown increased *CDH1* expression, and inhibited the repression of *CDH1* induced by TGF-β (Fig. 2A), which was consistent with immunofluorescence for E-cadherin (Fig. 1C). For *FN1*/*Fibronectin* whose expression was up-regulated by TGF-β, *EED* knockdown canceled the effect of TGF-β (Fig. 2B). These results suggested that *EED* knockdown inhibited the gene expression program of TGF-β-induced EMT in A549 cells.

During EMT process, it has been reported that the expression of E-cadherin is regulated by the transcriptional repressors such as ZEB1 and ZEB2 [10]. Thus we analyzed the expression of ZEB family transcription repressors in the *EED* knockdown cells. TGF-β treatment up-regulated the expression of *ZEB1* and *ZEB2* in A549 cells (Fig. 2C and D). *EED* knockdown slightly decreased the expression of *ZEB1* and *ZEB2*, but the reduction was statistically not significant. Instead, *EED* knockdown inhibited the TGF-β-dependent increase of both transcripts (Fig. 2C and D). This finding led us to investigate the possibility that the effect could be due to the regulation of *miR-200* family of microRNAs. The *miR-200* family has been reported to target and inhibit ZEB1 and ZEB2 specifically during the EMT process [19,20]. We examined whether *EED* knockdown would affect the expression of two representative miRNAs, *miR-200a* and *miR-200c*. Consistent with the previous reports [19], TGF-β treatment resulted in the decreased expression of *miR-200a* and *miR-200c* in A549 cells (Fig. 2E and F). *EED* knockdown increased their expression, and inhibited the down-regulation of both microRNAs induced by TGF-β (Fig. 2E and F).

We also analyzed the changes in protein expression for some of the EMT-related gene products in A549 cells. *EED* knockdown canceled TGF-β-dependent reduction of E-cadherin protein and increase of ZEB1 and ZEB2 proteins (Fig. 2G), which enabled us to confirm the QRT-PCR results. Next we tried to examine whether TGF-β signaling pathway would be impaired or not in the *EED* knockdown cells by detecting the phosphorylated SMAD3 proteins

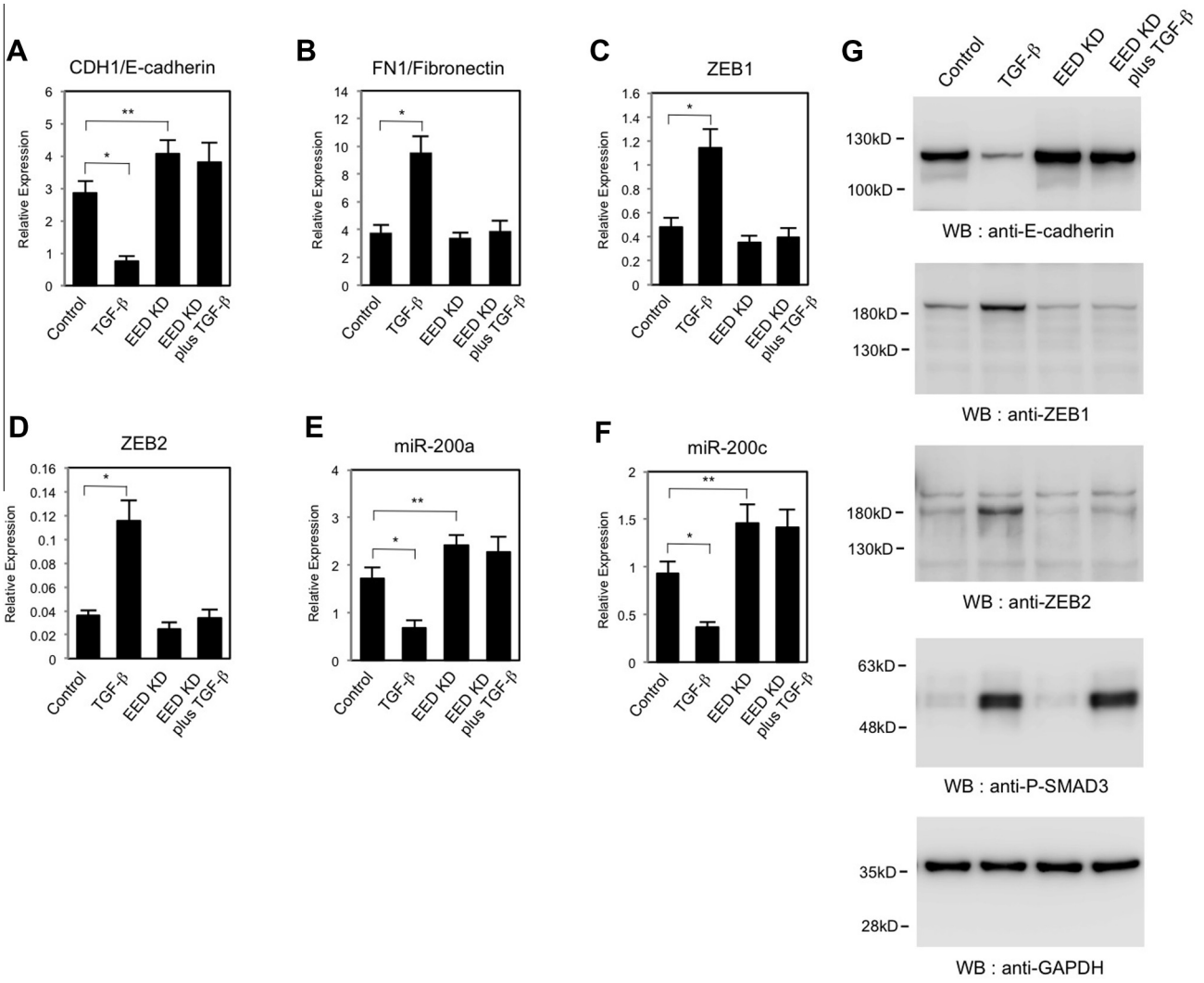

Fig. 2. Knockdown of *EED* affected the changes in expression of EMT-related genes induced by TGF-β. QRT-PCR analysis was performed to detect the expression of *CDH1*/*E-cadherin* (A), *FN1*/*Fibronectin* (B), *ZEB1* (C), *ZEB2* (D), *miR-200a* (E) and *miR-200c* (F) in control or *EED* knockdown A549 cells with or without treatment of TGF-β (*$P < 0.01$; **$P < 0.05$). (G) Western blotting was performed to detect the expression of E-cadherin, ZEB1, ZEB2 and phosphorylated SMAD3 (P-SMAD3) proteins using the corresponding antibodies. As a control, anti-GAPDH antibody was used to show that equal amounts of proteins were loaded.

after TGF-β treatment [9]. As shown in Fig. 2G, the phosphorylated SMAD3 proteins were induced by TGF-β and their levels were similar in control cells and *EED* knockdown cells. This result indicated that activation of the downstream SMAD3 transcription factor by TGF-β signal would not be impaired by *EED* knockdown. Moreover, we confirmed the effects of *EED* knockdown in the regulation of the EMT-related genes in another cancer cell line, HT29. QRT-PCR showed that *EED* knockdown similarly inhibited TGF-β-dependent changes of *CDH1*/*E-cadherin*, *FN1*/*Fibronectin*, *ZEB1*, *miR-200a* and *miR-200c* expression in HT29 cells (Supplementary Fig. S6). These results together suggested that EED might be directly involved in TGF-β-dependent transcriptional regulation of EMT-related genes in cancer cells.

3.3. EED is implicated in the transcriptional regulation of CDH1 and microRNA-200 family gene by TGF-β through the conversion of histone H3 methylation

EED is a core component of PRC2 enzyme complex that catalyzes methylation of K27 residue of histone H3 for transcriptional repression. Thus we examined the methylated status of histone H3 on the regulatory regions of *CDH1* and *miR-200* family genes, which were transcriptionally repressed during TGF-β-induced EMT, by ChIP assays. Genetically, the *miR-200* family is grouped in two polycistronic units: *miR-200b*/*200a*/*429* and *miR-200c*/*141* [21]. Following immunoprecipitation, the primer sets positioned upstream from the transcription start sites of *CDH1* gene and two microRNA clusters were used in quantitative PCR [7].

We first analyzed the transcriptionally repressive tri-methylated H3K27 (H3K27me3) status. On the regulatory regions of *CDH1*, *miR-200b*/*200a*/*429* and *miR-200c*/*141* genes, the levels of H3K27me3 were significantly increased after TGF-β treatment (Fig. 3), which was correlated with the transcriptional repression of these genes. On the other hand, transcriptionally active H3K4me3 marks decreased substantially on these regulatory regions by TGF-β (Fig. 3). More importantly, we observed the enhanced occupancies of EZH2, a catalytic subunit of PRC2, on these regulatory regions after TGF-β treatment (Fig. 3). These results suggested that TGF-β-induced recruitment of EZH2 on the regulatory regions might be responsible for the transcriptional repression. *EED* knockdown caused the decrease of H3K27me3, the increase of H3K4me3 and the decrease of EZH2 occupancies on these regions compared to control cells (Fig. 3), which was consistent with the observed increase of their expression (Fig. 2A, E

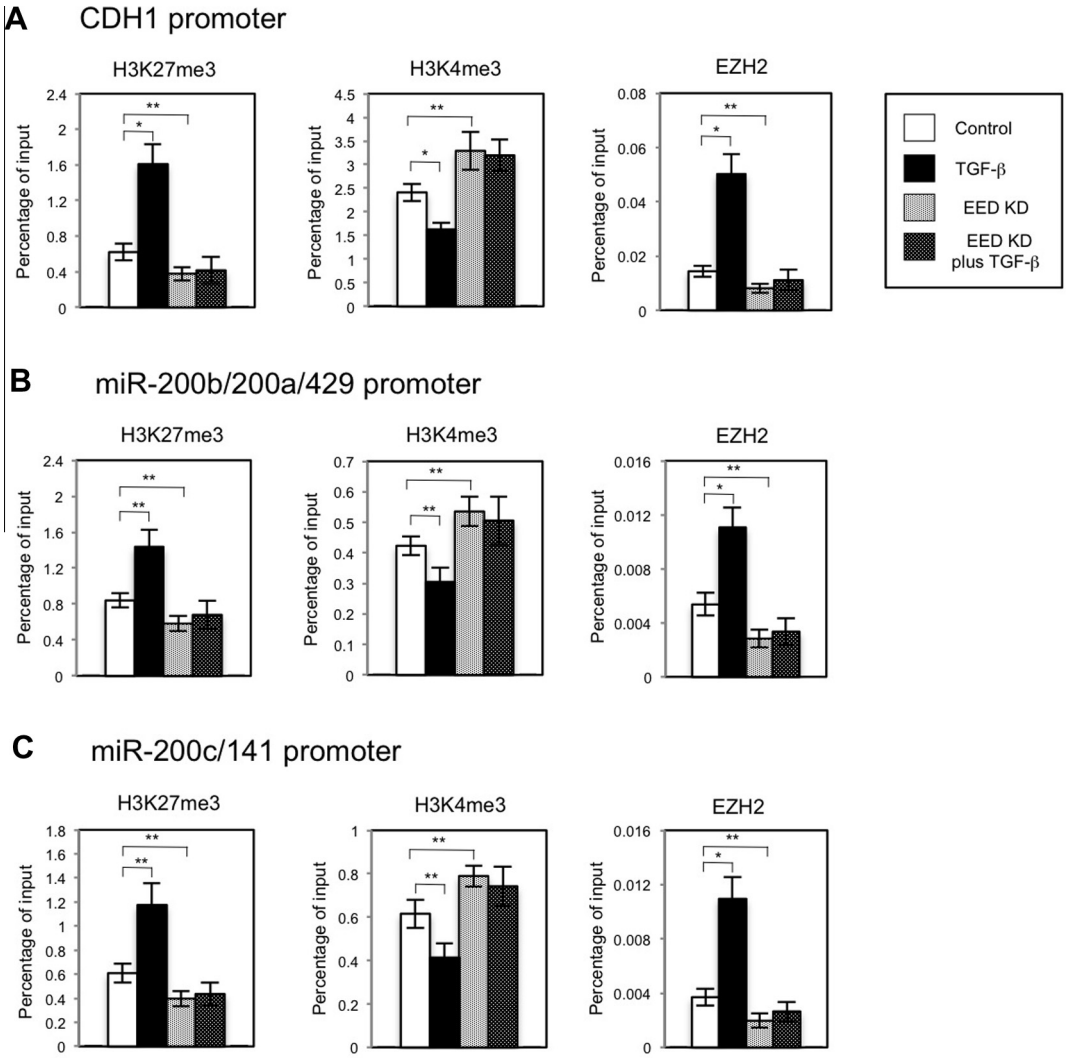

Fig. 3. Knockdown of *EED* affected the TGF-β-induced regulation of histone H3 methylation and EZH2 recruitment on the regulatory regions of *CDH1* gene and *miR-200* gene clusters. ChIP analyses of H3K27me3, H3K4me3 and EZH2 on the regulatory regions of *CDH1* (A), *miR-200b/200a/429* (B) and *miR-200c/141* genes (C) are shown. The occupancies of methylated histones or EZH2 protein on the regions were analyzed by QPCR (*$P < 0.01$; **$P < 0.05$).

and F). Moreover, *EED* knockdown inhibited TGF-β-induced changes of histone methylation and EZH2 occupancies on these regions (Fig. 3). These results suggested that endogenous EED was involved in TGF-β-induced transcriptional repression of *CDH1* and *miR-200* family genes during EMT and that its function was associated with the regulation of recruitment of EZH2 on the chromatin for histone methylation.

3.4. Over-expression of EED activated TGF-β-dependent transcriptional regulation leading to EMT

To extend our understanding for the regulation of EMT by EED, we examined the effects of *EED* over-expression in the cells. *EED* over-expression was confirmed by QRT-PCR and Western blot, and the level of over-expressed *EED* was not significantly changed before and after TGF-β treatment (Supplementary Fig. S7). Then we examined the expression of *CDH1/E-cadherin*, *FN1/Fibronectin*, *ZEB1*, *ZEB2*, *miR-200a* and *miR-200c* in the *EED* over-expressing cells. *EED* over-expression itself did not show any significant changes in the expression of EMT-related genes, but enhanced the effects of TGF-β in the expression of EMT-related genes (Fig. 4). In the *EED* over-expressing cells, the expression of *CDH1*, *miR-200a* and *miR-200c* was repressed more by TGF-β (Fig. 4A, E and F), and the expression of *FN1*, *ZEB1* and *ZEB2* was activated more by TGF-β (Fig. 4B, C and D). These results indicated that over-expression of *EED* potentiated TGF-β-dependent transcriptional regulation during EMT process.

Next we tried to examine whether *EED* over-expression would affect the methylated status of histone H3 and the occupancies of EZH2 on the regulatory regions of *CDH1*, *miR-200b/200a/429* and *miR-200c/141* genes by ChIP. On these regulatory regions, *EED* over-expression itself did not change the levels of H3K27me3 and H3K4me3 significantly, but enhanced the effects of TGF-β on both modifications (Fig. 4G and Supplementary Fig. S8), which was correlated well with the expression levels of *CDH1* and *miR-200* family genes. Furthermore, *EED* over-expression itself had little effect in EZH2 occupancies, but augmented EZH2 recruitment on these regulatory regions after TGF-β treatment. We could also detect the increased recruitment of FLAG-tagged EED proteins on the regions only in the presence of TGF-β (Fig. 4G and Supplementary Fig. S8). These results suggested that EED was directly involved in the recruitment of EZH2 on the regulatory regions of *CDH1*, *miR-200b/200a/429* and *miR-200c/141* genes for transcriptional repression, which was highly dependent on TGF-β signal.

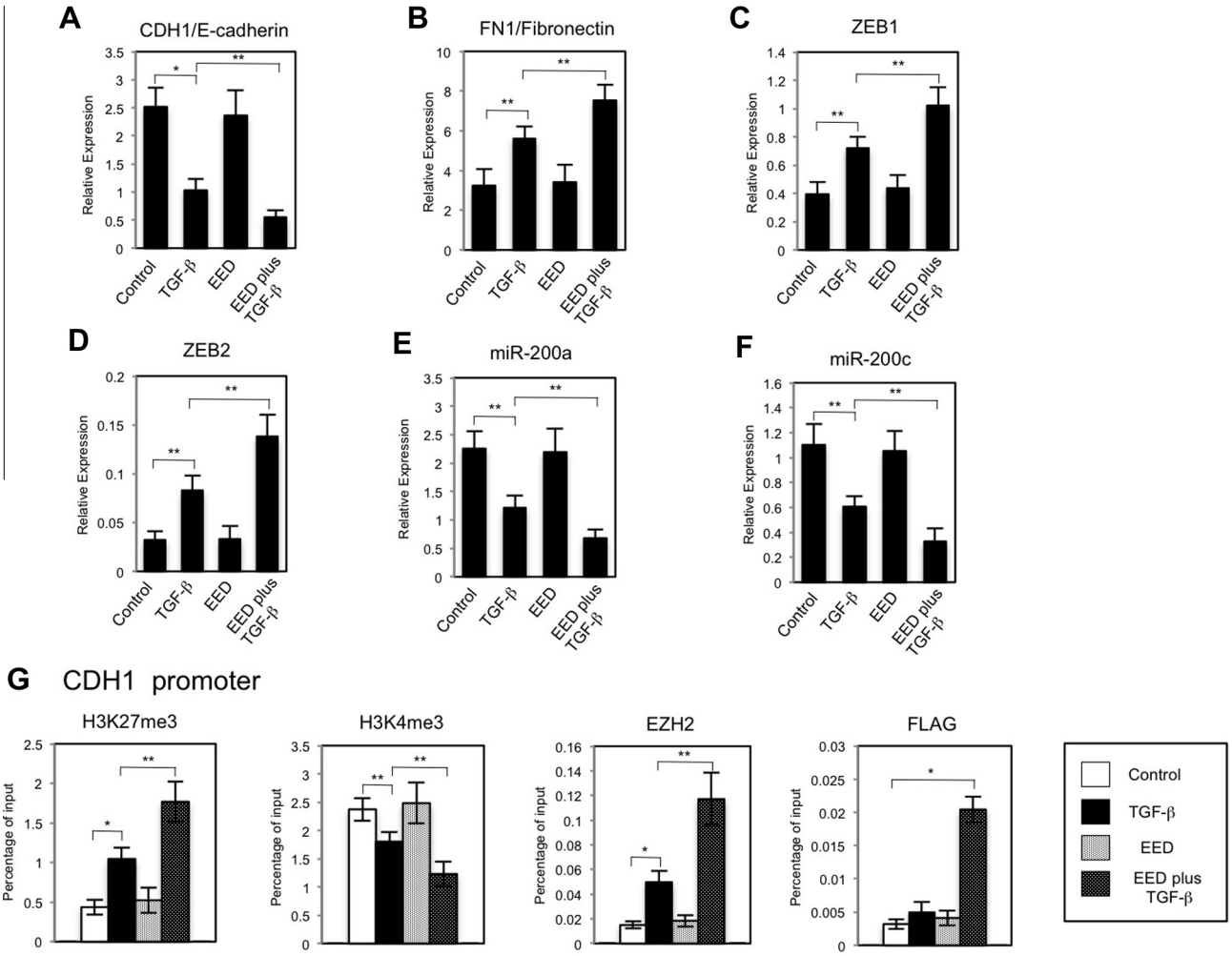

Fig. 4. Over-expression of *EED* enhanced the TGF-β-dependent changes in expression of EMT-related genes through the regulation of histone H3 methylation. QRT-PCR analysis was performed to detect the expression of *CDH1*/*E-cadherin* (A), *FN1*/*Fibronectin* (B), *ZEB1* (C), *ZEB2* (D), *miR-200a* (E) and *miR-200c* (F) in A549 cells infected with the control retrovirus or the retrovirus expressing *EED* with or without treatment of TGF-β ($^{*}P < 0.01$; $^{**}P < 0.05$). (G) ChIP analyses of H3K27me3, H3K4me3, EZH2 and FLAG-tagged EED on the regulatory regions of *CDH1* gene are shown ($^{*}P < 0.01$; $^{**}P < 0.05$).

4. Discussion

In this study, we found that knockdown of *EED* antagonized TGF-β-induced EMT by inhibiting TGF-β-dependent expression changes of EMT-related genes such as *CDH1*, *ZEB* family and *miR-200* family. On the other hand, overexpression of *EED* was shown to enhance the effects of TGF-β in their expression. ChIP analyses revealed that EED caused the increase of EZH2 occupancies and histone H3K27 methylation on the regulatory regions of *CDH1* and *miR-200* family genes in the presence of TGF-β, thereby leading to TGF-β-dependent transcriptional repression. Our study uncovers a novel role of EED, one of the core components of PRC2, in TGF-β-induced EMT of cancer cells.

Methylation of histone H3K27 is an important modification implicated in development, stemness and cancer [14]. The PRC2 complex regulates H3K27 methylation for gene silencing and EZH2 is its enzymatic component. Over-expression of *EZH2* was involved in tumor initiation and progression [16]. It was reported that EZH2 could repress the expression of *CDH1*/*E-cadherin* and *miR-200* family genes possibly through the regulation of H3K27 methylation [12,22]. Moreover, a genome-wide profiling of histone methylation during EMT revealed strong correlations between the dynamic changes of histone methylations and gene expression [23]. For certain target genes that have both modifications of H3K27me3 and H3K4me3, the level of transcription was dependent on the relative intensities of repressive H3K27me3 and active H3K4me3 marks. These previous findings were consistent with our results of ChIP experiments: TGF-β induced the increase of EZH2 occupancies and H3K27me3 and the decrease of H3K4me3 on the regulatory regions of *CDH1*, *miR-200b*/*200a*/*429* and *miR-200c*/*141* genes, resulting in transcriptional repression, but *EED* knockdown antagonized TGF-β-induced changes of EZH2 occupancies and histone methylations, consequently inhibiting TGF-β-dependent transcriptional repression. Therefore, this study validated the importance of H3K27 methylation during EMT program and demonstrated a novel mode of regulation for H3K27 methylation and PRC2 function mediated by EED.

Among the PRC2 core components, EED was found to be only one member whose expression was induced during TGF-β-induced EMT. We demonstrated that *EED* knockdown itself could increase the expression of *CDH1*, *miR-200a* and *miR-200c* possibly through the decrease of EZH2 occupancies and repressive H3K27me3 on their regulatory regions. These results suggest that endogenous EED is directly involved in the regulation of H3K27 methylation for the expression of EMT-related genes. Furthermore, *EED* knockdown inhibited TGF-β-induced EMT, indicating that EED is required for TGF-β-induced EMT of cancer cells. On the other hand, *EED* over-expression itself had little effect in the levels of EZH2

occupancies and H3K27 methylation on the target genes, but potentiated the effects of TGF-β in the expression of EMT-related genes through PRC2 recruitment. These results suggested that some additional factors and/or signals induced by TGF-β might be required for EED to regulate the recruitment and activation of PRC2 complex on the target genes. Currently, the activation mechanisms of EED and PRC2 during TGF-β-induced EMT remain elusive. Further studies will be required to extend our understanding.

Increasing evidence indicates that deregulation of enzymes and cofactors engaged in histone methylation has been associated with the initiation and progression of many human cancers [1–3]. Overexpression of EZH2 has been found in a number of tumors and correlated with poor prognosis [15,16]. However, there are few reports demonstrating the relationship between other PRC2 components and cancer development. A recent paper reported that up-regulation of *EED* expression was identified in breast cancer lymph node metastasis compared to primary tumors [24]. This result strongly supports our novel findings for the role of EED in cancer progression. In this study, we demonstrated that alteration of epigenetic regulation such as histone methylation and microRNA expression contributes to a critical step for malignant progression of cancer. Thus our study provides novel insights into potential epigenetic therapeutic strategies for the treatment of malignant tumors.

Acknowledgments

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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.2014.09.082.

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