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A multifunctional protein EWS regulates dermal development

EWS에 의한 피부 발달의 조절기작

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ABSTRACT

EWS (Ewing's Sarcoma) gene encodes a RNA/DNA binding protein that is ubiquitously expressed and involved in various cellular processes. EWS is a nuclear protein, whose nuclear localization is dependent upon its transactivating NH_2 terminus. Also, the EWS protein stimulates transcription mediated by the COOH terminal transactivation domain of the cofactor CREB-binding protein (CBP)/p300. EWS deficiency leads to impaired development and early senescence through unknown mechanisms. We found that EWS regulates the expression of Drosha and microRNAs. EWS deficiency resulted in increased expression of Drosha, a well known microprocessor, and increased levels of miR-29b and miR-18b. Importantly, miR-29b and miR-18b were directly involved in the posttranscriptional regulation of collagen IV alpha 1 (Col4a1) and connective tissue growth factor (CTGF) in EWS knock-out (KO) mouse embryonic fibroblast (MEFs) cells. The up regulation of Drosha, miR-29b and miR-18b and the sequential down regulation of Col4a1 and CTGF contributed to the

deregulation of dermal development in EWS KO mice. Otherwise, knock-down of Drohsa rescued miRNA-dependent down regulation of Col4a1 and CTGF proteins. Taken together, our data indicate that EWS is involved in posttranscriptional regulation of Col4a1 and CTGF via a Drosha-miRNA-dependent pathway. This finding suggests that EWS plays a novel role in dermal morphogenesis through the modulation of miRNA biogenesis.

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Keywords: EWS, Drosha, microRNA, Col4a1, CTGF, dermal development

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LIST OF ABBREVIATIONS

EWS Ewing's Sarcoma

Col4a1 collagen IV alpha 1

CTGF connective tissue growth factor

KO knock-out

MEFs mouse embryonic fibroblasts

TFIID transcription factor II D

CBP CREB binding protein

ECM extracellular matrix

miRNAs microRNAs

pri-miRNAs primary-miRNAs

pre-miRNAs precursor-miRNAs

RISC RNA induced silencing complex

Microprocessor miRNA processing machinery

qRT-PCR quantitative real time PCR

RNA-seq whole transcriptome sequencing

ChIP Chromatin Immunoprecipitation

LNA locked-nucleic-acid inhibitors

Anti-29b miR-29b inhibitor

Anti-18b miR-18b inhibitor

TET family of proteins TLS/FUS, EWS and TAF15

INTRODUCTION

Ewing sarcoma gene (*EWS*) is a member of the TET family proteins and encodes a RNA and single-strand DNA-binding protein. A role for EWS in RNA splicing has been implicated from its interactions with various splicing factors such as SF1/ZFM1, U1C, YB1, and TASR-1 and -2 (1). EWS also interacts with subunits of transcription factor II D (TFIID), CREB binding protein (CBP) and RNA polymerase II complexes (2), suggesting a role in basic transcription. Subsequent studies have shown that EWS acts as a transcriptional activator for BRN3A, HNF3 (3,4), and OCT4 (5) in a cell-type and promoter-specific manner. Interestingly, *EWS* gene is frequently rearranged by chromosomal translocations in several cancers, leading to its fusion with many transcription factors including FLI1, ATF1, WT1, and CHOP/GADD153. The resulting chimeric fusion proteins, such as EWS-FLI1, EWS-ATF1, and EWS-WT1, function as aberrant transcription factors that drive proliferation, survival and transformation (6). While much effort has been focused on the EWS-fusion

oncoproteins, very little effort has been invested on understanding the function of wild-type EWS. A recent study has shown that EWS has essential roles in precursor B lymphocyte development and meiosis (7). Furthermore, loss of *Ews* resulted in premature cellular senescence in mouse embryonic fibroblasts (MEFs) and in hematopoietic stem progenitor cells (8). Therefore, EWS is a multifunctional protein with roles in many different cellular processes.

Skin development is organized by complex and balanced mechanism of gene activation and silencing. Collagen IV is a major part of the dermal-epidermal junction and plays special roles in the maintenance of basement membrane integrity in the skin (9). Collagen IV family consists of six types, Col4a1, Col4a2, Col4a3, Col4a4, Col4a5, and Col4a6 (10). Previous studies demonstrated that reduced or depleted Collagen IV leads to impaired basement membrane stability (11). Connective tissue growth factor (CTGF) is a member of the CCN family of a cysteine-rich protein (12). CTGF induces cell adhesion and expression of the extracellular matrix protein collagen type I (13). Several studies have shown that the CTGF stimulates proliferation, angiogenesis, migration, extracellular

matrix (ECM) production, and cell attachment (14).

MicroRNAs (miRNAs) are a small noncoding RNAs (~22 nucleotides) that regulate gene expression at the posttranscriptional level. miRNAs can target mRNAs and induce degradation and translational repression. miRNA processing is initiated by RNA polymerase II as primary-miRNAs (pri-miRNA). Drosha cleaves pri-miRNA to precursor-miRNAs (pre-miRNAs) in the nucleus (15). Exportin-5 facilitates the exit of the pre-miRNAs and Dicer removes the loop of the pre-miRNAs to produce the mature miRNA duplex. One strand of the duplex is combined with the RNA induced silencing complex (RISC) (16). The miRNA processing machinery (microprocessor) including Drosha and DGCR8 may participate in the processing of miRNAs for the proper execution of gene expression programs during normal development, including skin development (17). The level of cell type-specific molecules and factors implicated in skin development are directly regulated by miRNAs. Dermal and epidermal development is organized by gene activation and silencing. Recent studies report that miRNAs have a quite important role in skin development (18).

EWS has been found in the complex containing Drosha by mass spectrometry analysis (19). However, the role of EWS in microRNA biogenesis or regulation has not been studied. To determine whether EWS has any roles in miRNA expression and/or regulation, we performed miRNA array and RNA-sequencing analyzing genome-wide data to identify which miRNAs and mRNAs are altered under EWS deficiency. We further validated targets and molecular pathways that are associated with the identified miRNAs *in vivo*. Our results indicate that EWS plays a crucial role in the expression of genes that are essential for the normal development of skin through the regulation of specific miRNAs.

MATERIALS AND METHODS

1. EWS MEF cell culture

EWS MEF cells were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA),100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen Life Tech, Grand Island, NY, USA) and 10% MEM Non-essential Amino acid solution (SIGMA, St Louis, MO, USA).

2. Subcellular Fractionation

EWS MEF cells were grown in a 10 cm dish and they were harvested in 450 ul of ice-cold buffer A (10mM HEPES at pH 7.9, 10 mM KCl, 1 mM dithiothreitol [DTT], and 0.1 mM EDTA at pH 8.0). EWS MEF cells dispersed by pipetting and incubated for 25 min on ice. Then 5 ul of 10% NP-40 was added, and cells were incubated for 2 min on ice. The nuclei were precipitated by centrifugation at 5000 rpm for 3 min at 4°C. The supernatant was taken as the cytoplsamic fraction.

3. Quantitative Real Time-PCR (qRT-PCR)

Total RNA was extracted from EWS MEF cells and NIH 3T3 by TRIzol reagent (MRC, Cincinnati, OH, USA). RNA was measured in a spectrophotometer at 260-nm absorbance. RNA analysis was conducted as follows. Fifty nanograms of RNA were used as a template for quantitative RT-PCR amplification, using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, OSK, Japan). Primers were standardized in the linear range of cycle before the onset of the plateau. Primer sequences are given in supplementary table1. Mouse GAPDH was used as an internal control. Two-step PCR thermal cycling for DNA amplification and real-time data acquisition were performed with an ABI StepOnePlus^{TM} Real-Time PCR System using the following cycle conditions: 95℃ for 1min x 1 cycle, and 95℃ for 15s, followed by 60℃ for 1 min x 40 cycles. Fluorescence data were analyzed by the ABI StepOnePlus software and expressed as C_t the number of cycles needed to generate a fluorescent signal above a predefined threshold. The ABI StepOnePlus software set baseline and threshold values.

4. Plasmid Construction

miR-29b (forward primer, 5'-CGCGGATCCAACTATTGCACGGACTTCAC-3' and reverse primer, 5'-CCGCTCGAGACACTGGACACTTACTTCAG-3') and miR-18b (forward primer, 5'-CGCGGATCCACCATGGTGATTTAATCAGA-3' and reverse primer, 5'-CCGCTCGAGCCGTTCAAATCATTTCTCAA-3') were amplified from EWS MEF cDNA by PCR. PCR product was cloned into pCDNA3 (Invitrogen Life Tech, Grand Island, NY, USA) with *BamH* I and *Xho* I (*NEW ENGLAND* BioLabs, Ipswich, MA, USA) restriction enzyme sites and sequenced. The 5' UTR of mouse Drosha promoter (-1248/+45) was amplified by PCR from EWS MEF genomic DNA (forward primer, 5'-CCGCTCGAGGTTTTGAAAACGCGTATTTG-3' and reverse primer, 5'-CCCAAGCTTCACGTATTGCCTTGCATCTC-3'). The PCR product was cloned into pGL4.14 vector (Promega) with *Kpn* I and *Xba* I (*NEW ENGLAND* BioLabs) restriction enzyme sites. The 3' UTR of mouse Col4a1 was amplified by PCR from EWS MEF cDNA (forward primer, 5'-CCGCTCGAGGAAGCCCACGCCATCCACCT-3' and reverse

primer, 5’ – CTAGTCTAGAGGGTTGGACAGCACTCACAT–3’). The 3’ UTR of mouse CTGF was amplified by PCR from EWS MEF cDNA (forward primer, 5’ – CCGCTCGAGTACAGTTATCTAAGTTAATT–3’ and reverse primer, 5’ – CTAGTCTAGATTCATATAAAAAATATATAT–3’). The PCR product was cloned into pmirGLO dual–Luciferase vector (Promega) with *Xho* I and *Xba* I (*NEW ENGLAND* BioLabs) restriction enzyme sites.

5. RNA interference experiments and Western blot analysis

30 nM of siRNA duplex were transfected in NIH 3T3 and EWS KO MEF cells with RNAiMax transfection reagent (Invitrogen Life Tech, Grand Island, NY, USA) according to the manufacturer’s instructions. siRNAs and inhibitor of miR–29b and miR–18b were synthesis from COSMO GENETECH, Seoul, Korea. Target sequences of mouse siDrosha, siDGCR8, and siDicer are 5’ –AGAUCACCGUCUCUAGAAA–3’, 5’ –AACAAUUUGGAGCUAGAUGAA–3’, and 5’ –ACACAGCAGUUGUCCUAAA–3’. Inhibitor miR–29b sequences are 5’ –UAAACCACCAUAUGAAACCAGC–3’ and miR–18b sequences are 5’ –

CUAACAGCACUAGAUGCACCUUA-3'. EWS MEF and NIH 3T3 cells were collected at 72 hr after siRNA and Flag-EWS transfection. The cells were dispersed by pipetting in lysis buffer (10 mM Tris at pH 7.4, 1 mM ethylenediaminetetra acetic acid [EDTA] at pH 8.0 500 mM NaCl, and 0.5% Triton X-100) and incubated for 30 min on ice. Primary antibodies used in this study are rabbit anti-Col4a1 antibody (Upstate Biotechnologies and abCam, Cambridge, ENG, United Kingdom), rabbit anti-CTGF antibody (Upstate Biotechnologies and abCam, Cambridge, ENG, United Kingdom), rabbit anti-Drosha antibody (Upstate Biotechnologies and abCam), rabbit anti-DGCR8 antibody against recombinant DGCR8 protein prepared in *E. coli* (16), goat anti-DGCR8 antibody (Upstate Biotechnologies and abCam, Cambridge, ENG, United Kingdom), rabbit anti-Dicer (Santa Cruz Biotechnology, Dallas, Texas, USA), rabbit anti-Ago2 (Santa Cruz Biotechnology, Dallas, Texas, USA), mouse anti-EWS (Santa Cruz Biotechnology, Dallas, Texas, USA), mouse anti-Flag (SIGMA, St Louis, MO, USA), mouse anti β-actin and mouse anti-tubulin (Millipore, Billerica, MA, USA).

6. Confocal microscopy

Immunofluorescence staining and confocal microscopy was used to determine mouse anti-EWS (Santa Cruz Biotechnology, Dallas, Texas, USA). Images were analyzed using a spinning disk confocal microscope (Olympus DSU, Shinjuku, Tokyo, Japan). Deconvolution and 3-dimentainal construction of the confocal image was performed by AQI-X-COMBO-CWF program (Media cybernetics Inc, Rockville, MD, USA). Control experiments were performed in the absence of primary antibody or in the presence of blocking peptide.

7. Whole transcriptome sequencing (RNA-seq) and gene analysis

For the mRNA-Seq sample preparation, the Illumina standard kit was used according to the manufacturer's protocol. Briefly, 3 μg of each total RNA sample was used for polyA mRNA selection using streptavidin-coated magnetic beads, followed by thermal mRNA fragmentation. The fragmented mRNA was subjected to cDNA synthesis using reverse transcriptase (SuperScript II, Grand Island, NY, USA) and random primers.

The cDNA was further converted into double stranded cDNA and, after an end repair process (Klenow fragment, T4 polynucleotide kinase and T4polymerase), was finally ligated to Illumina paired end (PE) adaptors. Size selection was performed using a 2% agarose gel, generating cDNA libraries ranging in size from 200 250 bp. Finally, the libraries were enriched using 10 cycles of PCR and purified by the QIAquick PCR purification kit (Qiagen, Venlo, Limburg, Netherlands). The enriched libraries were diluted with Elution Buffer to a final concentration of 10 nM. Each library was run at a concentration of 8 pM on one Genome Analyzer (GAIIx) lane using 53 bp sequencing. Reads were then processed and aligned to the mouse genome UCSC build mm9 using GSNAP [24]. GSNAP uses the normalized RNA-Seq 8 fragment counts to measure the relative abundances of transcripts. The unit of measurement is Reads Per Kilobase of exon per Million fragments mapped (RPKM) (25).

8. Functional enrichment analysis and network analysis

For the functional enrichment analysis we selected genes whose RPKM levels in EWS KO (-/-) MEFs were three times

smaller than in EWS WT (+/+) MEFs. 520 genes were selected and grouped in terms of GO biological processes (GOBPs) and KEGG pathway using DAVID (26). Four groups by GOBPs were selected: (1) development, (2) cell adhesion, (3) cell proliferation & cytoskeleton organization, (4) morphogenesis. Then direct neighbors of the 91 genes were collected by querying each of the 91 genes to the NCBI Entrez EUtilities Web Service Client provided by Cytoscape (v. 2.8.0) (27) and STRING (28). Finally, 46 genes were selected by removal of some genes without neighboring and were displayed in a network with 161 genes including neighbors by using Cytoscape(v. 2.8.0). Genes in the network were grouped according to their interaction and function.

9. Transcriptional Profile of miRNA Processing Genes

To visualize heat map transcriptional profiles of miRNA processing genes, we collected miRNA processing-related genes from the NCBI Entrez E-utility Web Service using Cytoscape (v 2.8.0). 17 miRNA processing related genes with RPKM>=1.0 were finally selected. The mRNA expression levels of miRNA processing-related genes in EWS WT (+/+)

and EWS KO (−/−) were plotted in a heat map using *gplots* package in R Development Core Team.

10. Histopathological Evaluation

Serially cut skin tissue sections were immunostained for Col4a1 (Upstate Biotechnologies and abCam) and CTGF (Upstate Biotechnologies and abCam) using a previously reported conjugated secondary antibody method in skin tissue samples. Preabsorption with excess target proteins, omission of the primary antibodies, and omission of secondary antibodies were performed to determine the amount of background generated from the detection assay.

11. Statistical Analysis

The data are presented as the mean ± standard error of the mean (SEM). Data analysis was performed by Student's *t* test or one−way ANOVAs followed by Mann−Whitney and Kruskal−Wallis tests. Differences were considered statistically significant when $p<0.05$.

12. Subcellular fraction

Harvested EWS MEF cells were washed with PBS and centrifuged at 1000 rpm for 4 min at 4 ℃. The cell pellet was resuspended in 500 ㎕ of ice-cold Buffer (10 mM HEPES-KOH, pH7.9, 1.5 mM MgCl2, 10 mM KCL, 0.5 mM DTT and protease inhibitors), kept on ice for 5 min and homogenized twenty times using a tight Dounce pestle. The homogenized sample was centrifuged at 1000rpm for 5min at 4 ℃ to separate the nucleus. The supernatant was retained as the cytosolic fraction. The pellet was resuspended in 300 ㎕ 0.25 M sucrose buffer (containing 10 mM MgCl2) and layered over 300 ㎕ 0.35 M sucrose buffer (0.5 mM MgCl2) and centrifuged at 2500 rpm for 5 min at 4 ℃. This step resulted in a separation of cleaner nuclear fraction.

13. Drosha promoter activity analysis

The Drosha promoter analysis was performed using (luciferase) vector (-1289/+45). pGL4.14-Dorsha reporter were transiently transfected in NIH 3T3 cells with or without EWS vector. The luciferase activity was measured 72 hr after the transfection. The promoter activity was normalized to

protein concentrations or *Renilla* luciferase activity. The 3' UTR of Col4a1 and CTGF analysis was performed using (pmirGLO dual-luciferase vector). pmirGLO-Col4a1 and CTGF reporter were transiently transfected in NIH 3T3 cells with miR-29b and miR-18b. The luciferase activity was measured 48 hr after the transfection and normalized using Dual-Luciferase Reporter System (Promega) according to the manufacturer's instruction.

14. Chromatin Immunoprecipitation (ChIP)

ChIP for EWS binding to DNA was performed using a ChIP assay kit (Santa Cruz Biotech, Santa Cruz). The cells were cross-linked with 1 % formaldehyde for 10 min at room temperature. The lysates were sonicated ten times with each time for 30 s using Bioruptor (Diagenode). After centrifugation, the supernatant was diluted in ChIP dilution buffer and then incubated overnight at 4 ° C with the primary antibody. The eluted DNA was quantified by qPCR and normalized to IgG control.

15. Northern blot analysis of small RNAs

Total RNA was isolated from EWS MEF cells with TRIzol reagent (MRC). 35 micro gram of total EWS MEF RNA were separated on 15% urea-polyacrylamice gel and transferred to Biodyne nylon transfer membranes (Pall Co.). Oligonucleotides complementary to miR-29b; 5'-AACACTGATTTCAAATGGTGCTA-3' and miR-18b; 5'-CTAACAGCACTAGATGCACCTTA-3' were end labeled with T4 polynucleotide kinase (*NEW ENGLAND* BioLabs) and used as probes.

RESULTS

EWS modulates miRNA biogenesis.

In the first series of experiments, we performed a microarray analysis of miRNA expression to determine the role of EWS in miRNA biogenesis using EWS WT (+/+) and KO (−/−) MEF cells (Figure 1A). We found upregulated versus downregulated miRNAs in the absence of EWS. We chose to focus our studies on the regulation and function of miR−29b and miR−18b in the context of EWS deficient conditions because they are known target mRNAs related to the formation of extracellular matrix (ECM), specifically collagen IV alpha 1 (Col4a1) and connective tissue growth factor (CTGF), (7, 20). Furthermore, levels of miR−29b and miR−18b are significantly elevated in EWS KO mice, which have abnormal skin development (Table 1 and Figures 2A and B). We performed quantitative real time PCR (qRT−PCR) analysis of miR−29b and miR−18b and confirmed that both miRNAs are highly and significantly induced in EWS KO cells (Figure 1C). We proposed that the increase of mature miRNAs is likely mediated through the activity of

microprocessor via subsequent processing of pri-miRNA in the EWS KO cells (Figure 1B) (15). In this regard, to test whether mature forms of miR-29b and miR-18b are derived from pri-forms of miRNAs, we measured the levels of pri-miR-29b and pri-miR-18b in subcellular fractions (Figure 1B). As we expected, the levels of pri-miR-29b and pri-miR-18b were significantly decreased in the nuclear factions of EWS KO cells. To further confirm whether the processing of miR-29b and miR-18b are regulated by EWS, we ectopically expressed EWS and measured miR-29b and miR-18b levels (Figure 1D). Overexpression of EWS significantly reduced miR-29b and miR-18b levels. This result shows that EWS regulates the processing of miR-29b and miR-18b.

miR-29b and miR-18b negatively regulates Col4a1 and CTGF expression.

In order to examine whether proposed targets of miRNAs are altered at the transcript level and what other profiles of transcriptome (mRNA) are modulated under EWS deficiency condition, we carried out the whole transcriptome sequencing (RNA-seq) and gene analysis. We found that dermal

development related gene *Col4a1* and *CTGF*, target of miR-29b and miR18b, and other genes (*FGF10, Col8a1, DMPK, FGFR2, MGP, MYOM1, ASPN, ENG, PRRX1* and *Serpinb1a*) were altered in EWS KO cells in comparison to EWS WT cells (Figures 3A and B). We further performed functional enrichment analysis for the transcriptome data and found that 46 genes among the 161 genes are mainly involved in processes associated with cell adhesion, cell proliferation and cytoskeleton organization, development and morphogenesis. Then, we generated a biological network to define relationships among 46 genes and their specified processes as shown in Figure 4A. The network showed dense connections between the nodes associated with the processes in the network, indicating EWS deficiency deregulates genes that are closely linked to cell proliferation and impairs development and morphogenesis. Col4a1 and CTGF protein levels were significantly decreased in EWS KO cells (Figure 4B). qRT-PCR analysis confirmed that the mRNA levels of Col4a1 and CTGF also were dramatically decreased in EWS KO cells (Figure 4C). These results, along with a fact that EWS is primarily localized in the nucleus and has presumptive roles in transcription, suggest that the

expression of Col4a1 and CTGF might be regulated at the posttranscriptional level. To test this, we performed RT-PCR with RNAs isolated from nuclear and cytoplasm of EWS WT and KO cells. Amplification of Col4a1 (first and second) and CTGF (first) introns were markedly decreased in the nucleus and the cytoplasm of EWS KO cells, which is consistent with reduced posttranscription of Col4a1 and CTGF in EWS KO cells (Figure 5D). Amplification of Col4a1 and CTGF exons in EWS KO cells were also lower than those of EWS WT cells (Figure 4D). We also examined whether Col4a1 and CTGF transcripts are regulated posttranscriptionally by EWS deficiency (Figure 4D). qRT-PCR showed that levels of Col4a1 and CTGF mRNA are significantly reduced in the cytoplasmic fraction and only slightly decreased in the nuclear fraction of EWS KO cells. This suggests that EWS in involved in transcriptional regulation in part and more substantially in posttranscriptional regulation of Col4a1 and CTGF. Since we were interested in determining how of Col4a1 and CTGF mRNA levels are reduced in the cytoplasmic fraction given that miRNA (miR-29b and miR-18b) levels are significantly expressed in EWS KO cells, we chose to study posttranscriptional regulation of Col4a1 and CTGF

through miRNA in the context of EWS deficiency. To verify whether miR-29b and miR-18b target and regulate Col4a1 and CTGF mRNA, we overexpressed miR-29b and miR-18b and measured the protein levels of Col4a1 and CTGF, respectively. miR-29b and miR-18b markedly decreased the protein levels of Col4a1 and CTGF (Figure 4F). qRT-PCR results showed that the reduction of Col4a1 and CTGF mRNAs are inversely correlated with high levels of ectopically-expressed miR-29b and miR-18b (Figure 4E).

To investigate whether EWS directly affects the expression of Col4a1 and CTGF, we ectopically expressed EWS and measured the transcript and protein levels of Col4a1 and CTGF. EWS increased mRNA and protein levels of Col4a1 and CTGF in EWS WT and KO MEFs (Figures 5A and B). While Col4a1 and CTGF protein levels were robustly reduced in EWS KO MEFs in which the levels of miR-29b and -18b were elevated, the ectopic overexpression of miR-29b and -18b only moderately down regulated Col4a1 and CTGF protein levels. The mechanism responsible for this discrepancy is unclear but it in any case it seems that endogenously expressed miR-29b and -18b are more efficient posttranscriptional

regulators that their exogenous counterparts in EWS KO MEFs. The underlying causes of this differential will require further investigation.

EWS regulates Drosha expression.

Since we discovered that Col4a1 and CTGF are regulated by abnormal increases of miR-29b and miR-18b under EWS deficiency, we proposed that microprocessor, the miRNA processing machinery, might also be affected and they are consequently involved in the generation of mature miRNAs in EWS MEF cells. In this context, we performed functional enrichment analysis for the transcriptome data to find out the expression of miRNA processing-related genes including microprocessors (Drosha, DGCR8, and Dicer). The heat map showed alterations of miRNA processing-related genes (Figure 6A). Interestingly, we found that both mRNA and protein level of Drosha were highly and significantly elevated in EWS KO cells (Figures 6B and C). The protein levels of DGCR8 and Dicer did not show significant change in EWS KO cells (Figure 6C). Considering a fact that nuclear localization of Drosha protein is important in the miRNA processing, we further

performed subcellular fractionation and identified that Drosha is highly increased in the nucleus of EWS KO cells in comparison to EWS WT cells (Figure 7A). In consistent with the fractionation data, the immunofluorescence staining and confocal microscopy showed that the strong immunoreactivity of Drosha is found in nucleus in EWS KO cells while the immunoreactivity of DGCR8 is barely increased in the nucleus (Figure 6D). To examine whether EWS directly affects the transcription of Drosha, we performed a Drosha promoter assay and Chromatin Immunoprecipitation (ChIP). Overexpression of EWS repressed the transcriptional activity of Drosha promoter in a dose dependent manner and EWS-DNA occupancy within the region of the Drosha promoter was increased in NIH 3T3 cells (Figures 8A and B). In addition, the protein levels of Drosha was consistently decreased by EWS in a dose dependent manner while the protein levels of DGCR8 and Dicer was not changed by EWS (Figure 6E). To study whether the level of Drosha is directly regulated by EWS, we ectopically expressed EWS and measured the mRNA and protein levels of Drosha in EWS WT and KO MEFs. Both mRNA and protein levels of Drosha were downregulated by EWS while DGCR8 and

Dicer levels were not affected (Figures 6F and G). As we expected, the level of Drosha mRNA was inversely correlated with the level of EWS (Figures 8C and D). These data indicate that EWS modulates the expression of Drosha specifically among several microprocessors that are involved in the biogenesis of miRNAs.

Drosha alters miR-29b and miR-18b processing and involves in Col4a1 and CTGF expression.

In order to identify the expression of pre- and mature forms of miR-29b and miR-18b in EWS KO MEFs, we further carried out Northern blot analysis. The levels of mature miR-29b and miR-18b were markedly elevated in EWS KO MEFs (Figure 9A). To verify whether Drosha plays an effecter role for the biogenesis of miR-29b and miR-18b, we determined pri- and mature forms of miR-29b and miR-18b by RT-PCR and qRT-PCR after the induction of Drosha or the knock down of Drosha. Overexpression of Drosha significantly decreased the levels of pri-miR-29b and pri-miR-18b in a dose dependent manner (Figures 9B and C). qRT-PCR analysis verified that Drosha reduces the level of pri-miR-29b in dose dependent manner.

In addition, Drosha increased the level of miR-29b in dose dependent manner. This data indicated that Drosha directly regulates the processing of pri-miR-29b to mature forms of miR-29b (Figure 9D). In contrast, knock down of Drosha significantly restored the level of pri-miR-29b (Figure 9E).

To prove a hypothetical pathway that the alteration of Drosha activity under EWS deficiency subsequently contributes to miRNA-mediated posttranscriptional regulation of Col4a1 and CTGF, we applied RNAi technique and observed the effect of microprocessor depletion on the level of Col4a1 and CTGF. When we knock down Drosha, DGCR8, and Dicer with verified siRNAs, the protein levels of DGCR8 was slightly reduced. This is caused by off-target effects (15). The protein levels of Col4a1 and CTGF were specifically increased by the depletion of microprocessors in EWS MEFs (Figure 9F). We also confirmed increases in Col4a1 and CTGF mRNA levels by knocking down of Drosha, DGCR8, and Dicer, which demonstrates that microprocessor-dependent miRNA pathway involves in the posttranscriptional regulation of Col41 and CTGF the in the cytoplasm (Figures 10A and B). Furthermore, we addressed whether the regulatory mechanism of Col4a1 and

CTGF found in EWS KO cells is reproducible in an established fibroblast cell line such as NIH 3T3 cells. As expected, overexpression of EWS induced the expression of Col4a1 and CTGF (Figure 11A). Consistent with our previous results, expression of EWS led to a decrease in Drosha protein level (Figure 11A), and an increase of Col4a1 and CTGF mRNA levels in NIH 3T3 cells (Figure 11B). The mRNA levels of Drosha were also decreased by EWS in NIH 3T3 cells (Figure 11B). Otherwise, knock-down of the microprocessor increased mRNA and protein levels of Col4a1 and CTGF in NIH 3T3 as similar to the results in EWS MEF cells (Figures 11C and D). When miR-29b and miR-18b were highly expressed, both mRNA and protein levels of Col4a1 and CTGF were decreased in NIH 3T3 cells (Figure 12A). The exogenous induction of miR-29b and miR-18b in NIH 3T3 cells was confirmed by qRT-PCR (Figures 12B and C). To further verify the target specificity of miR-29b and miR-18b against Col4a1 and CTGF, we performed luciferase reporter assay using 3' UTR of Col4a1 and CTGF in NIH 3T3 cells. The posttranscriptional regulation of Col4a1 and CTGF was significantly down regulated by miR-29b and miR-18b, respectively (Figures 12D and E). We also

carried out locked-nucleic-acid inhibitors (LNA) experiments to decrease miR-29b and miR-18b. Both miR-29b inhibitor (anti-29b) and miR-18b inhibitor (anti-18b) markedly increased Col4a1 and CTGF protein and mRNA levels and decreased miR-29b and miR-18b levels in NIH 3T3 cells (Figures 12F and G).

These findings support that the alteration of microprocessor activity by EWS contributes to miRNA-mediated posttranscriptional regulation of Col4a1 and CTGF.

EWS deficiency upregulates Drosha, miR-29b, and miR-18b and downregulates Col4a1 and CTGF *in vivo*.

To examine whether *in vitro* findings on the role of EWS in the regulation of Drosha, miR-29b and miR-18b, and Col4a1 and CTGF are reproducible *in vivo*, we detected their mRNA and protein levels in the skin of EWS WT and KO mice. The mRNA level of Drosha was significantly increased in the skin of EWS KO mice (Figure 13A). In addition, the protein level of Drosha was significantly increased in the skin tissues of EWS KO mice while proteins levels of DGCR8 and Dicer were not altered (Figure 13D). Importantly, the expression levels of miR-29b

and miR-18b were significantly increased in the skin of EWS KO mice (Figure 13B). qRT-PCR analysis confirmed that the mRNA levels of Col4a1 and CTGF were significantly downregulated in the skin of EWS KO mice (Figure 13C). The protein levels of Col4a1 and CTGF were significantly lower in the skin of EWS KO mice than in EWS WT mice (Figure 13D). These results indicate that EWS deficiency deregulates the expression of Col4a1 and CTGF by altering Drosha-miRNA-dependent posttranscription. The Nissl staining represented abnormal development in the skin of EWS KO mice in comparison to EWS WT mice (Figure 13E). The immunohistochemistry showed that both immunoreactivity of Col41a and CTGF were markedly reduced in the skin of EWS KO mice (Figure 13F).

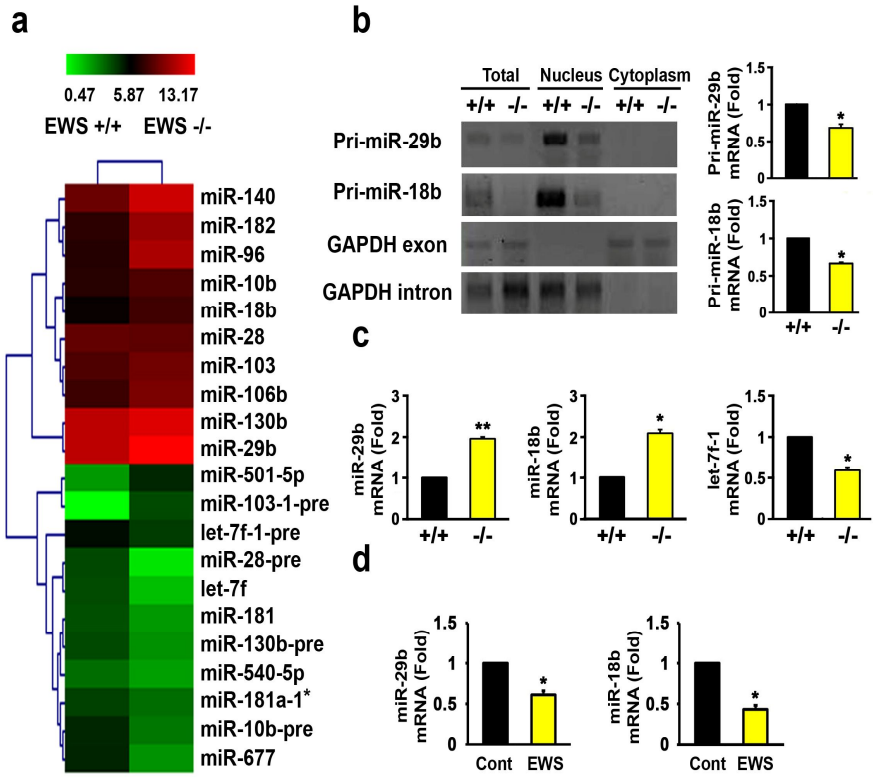

Figure 1 EWS deficiency alters the level of miRNAs.

(A) The heat map of miRNA array showed that relative expressions of miR-29b and miR-18b are highly increased in EWS KO MEF. The relative expression of miRNAs is displayed as colors: higher (red) or lower (green). The heat map represents the average of two samples. (B) Reverse transcriptase PCR (RT-PCR) analysis verified that the levels of pri-miR-29b and pri-miR-18b are decreased in subcellular fractions of EWS KO MEF compared to EWS WT MEF. The

intron and exon of mouse GAPDH were amplified as markers of nuclear and cytoplasmic RNA, respectively. The graphs (right panel) represent the average ± SEM of three separate experiments. (C) Quantitative real-time PCR (qRT-PCR) analysis verified that miR-29b and miR-18b are significantly increased in EWS KO MEF. Let-7f-1 was decreased in EWS KO MEF. The data represent the average ± SEM of three separate experiments. (D) qRT-PCR analysis showed that overexpression of EWS significantly reduces miR-29b and miR-18b levels in EWS KO MEFs. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$.

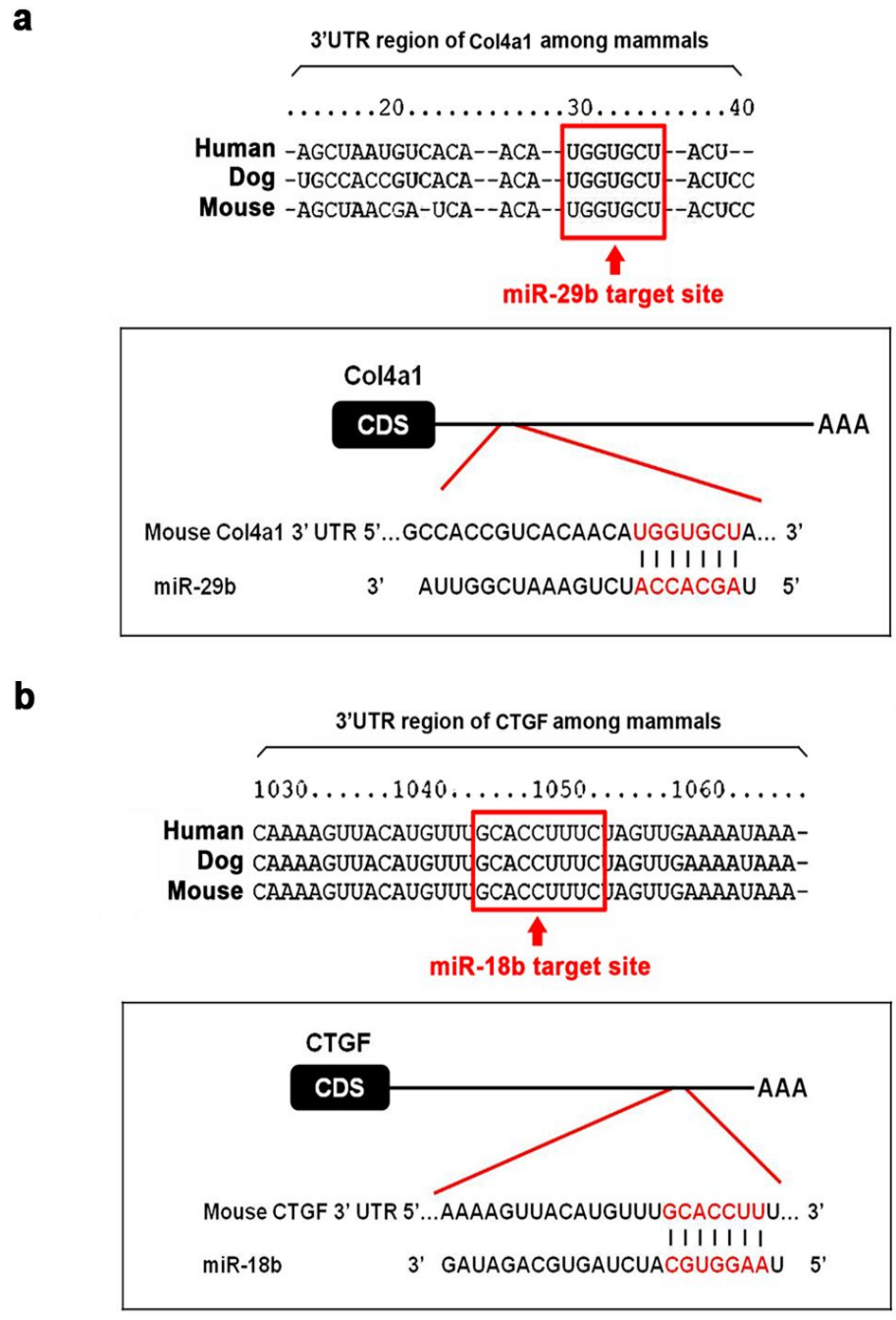

Figure 2. Col4a1 and CTGF are targeted by miR-29b and miR-18b, respectively.

(A) The homology of miR-29b target sequences on the 3' UTR of Col4a1 is conserved among mammals (upper panel). A schematic diagram explains consensus base pairing between miR-29b with the 3' UTR sequences of mouse Col4a1 (bottom panel). (B) The homology of miR-18b target sequences on the 3' UTR of CTGF is conserved among in mammals (upper panel). A schematic diagram shows consensus base pairing between miR-18b with the 3' UTR sequences of mouse CTGF (bottom panel). The identification of miR-29b and miR-18b target sequences was analyzed by TargetScan (http://www.targetscan.org).

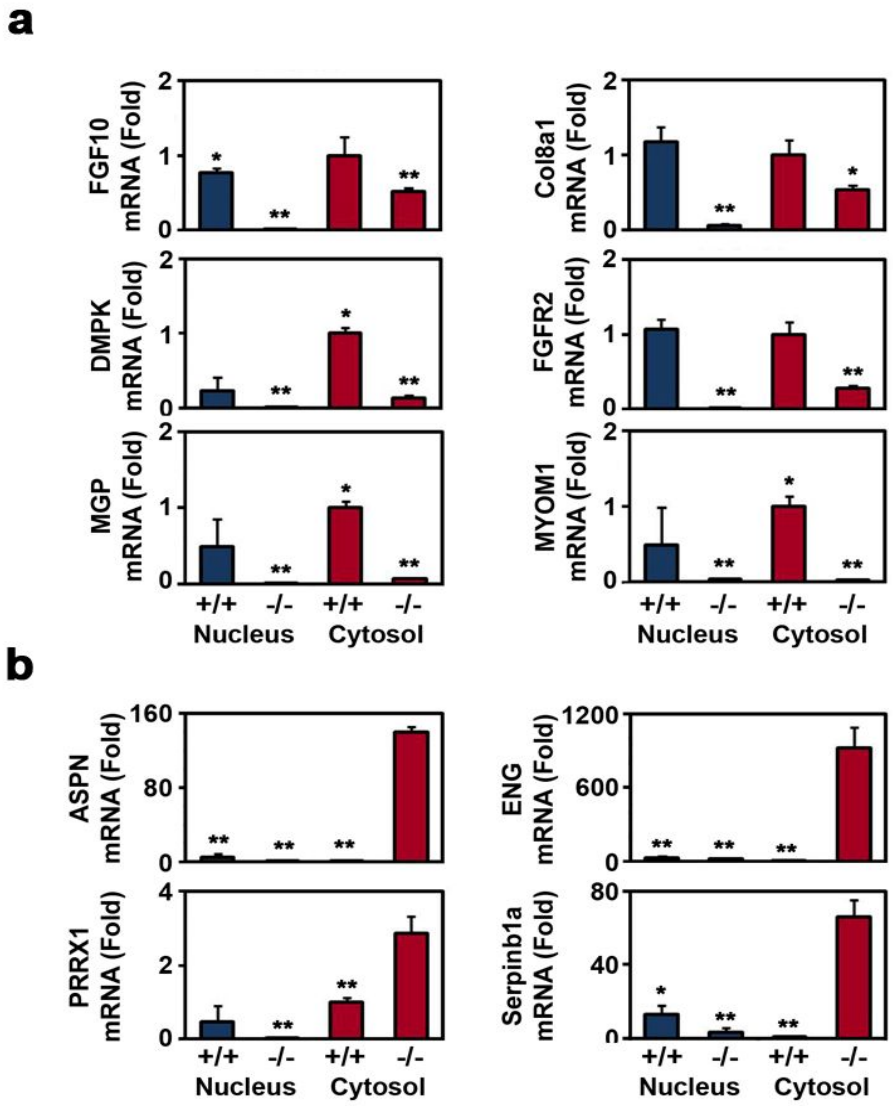

Figure 3. EWS deficiency leads to alteration of gene expression associated with development, morphogenesis, cell adhesion, and cell death.

(A) The mRNA levels of FGF10, Col8a1, DMPK, FGFR2, MGP, and MYOM1 were downregulated in EWS MEF cells. The RNAs isolated

from cytosolic and nuclear fraction were analyzed by reverse transcriptase-PCR (RT-PCR). The data represent the average ± SEM of three separate experiments. (B) The mRNA levels of ASPN, ENG, PRRX1 and Serpinb1a was upregulated in EWS MEF cells. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$.

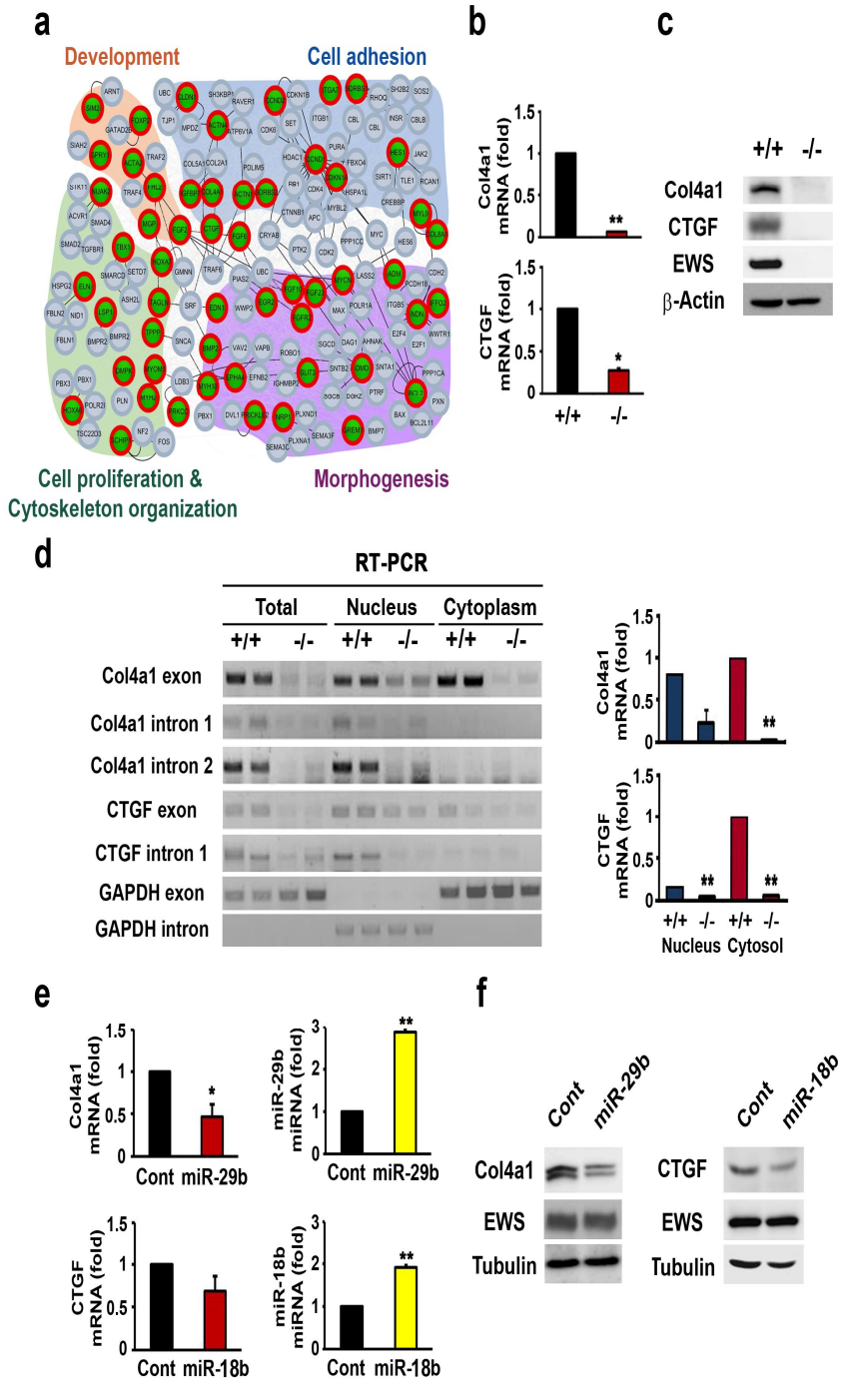
a
Development
Cell adhesion
Cell proliferation &
Cytoskeleton organization
Morphogenesis
b
Col4a1 mRNA (fold)
CTGF mRNA (fold)
+/+
-/-
c
+/+ -/-
Col4a1
CTGF
EWS
β-Actin
d
RT-PCR
Total
Nucleus
Cytoplasm
+/+ -/- +/+ -/- +/+ -/-
Col4a1 exon
Col4a1 intron 1
Col4a1 intron 2
CTGF exon
CTGF intron 1
GAPDH exon
GAPDH intron
Col4a1 mRNA (fold)
CTGF mRNA (fold)
+/+ -/- +/+ -/-
Nucleus Cytosol
e
Col4a1 mRNA (fold)
Cont miR-29b
miR-29b miRNA (fold)
Cont miR-29b
CTGF mRNA (fold)
Cont miR-18b
miR-18b miRNA (fold)
Cont miR-18b
f
Cont
miR-29b
Col4a1
EWS
Tubulin
Cont
miR-18b
CTGF
EWS
Tubulin

Figure 4. Col4a1 and CTGF are targeted by miR-29b and miR-18b under EWS deficiency condition.

(A) A biological network delineating cellular processes associated with cell adhesion, cell proliferation and cytoskeleton organization, development and morphogenesis is represented by functionally enriched 46 genes. Node colors denote the down regulation (green with red circle) or no change (gray) of mRNA expression under EWS deficiency. Genes in the network were grouped according to their interaction and function. The lines represent activation information obtained from KEGG pathways. (B) Quantitative real time-PCR (qRT-PCR) analysis verified that the mRNA levels of Col4a1 and CTGF were significantly reduced in EWS KO MEF. The data represent the average ± SEM of three separate experiments. (C) Western blot analysis confirmed that Col4a1 and CTGF were downregulated in EWS KO MEF. (D) Reverse transcriptase-PCR (RT-PCR) analysis verified that mRNAs of Col4a1 (exon, intron1 and 2) and CTGF (exon and intron 1) the Col4a1 and CTGF are differently moldulated in subcellular fractions of EWS WT and KO MEFs. The intron 1 and exon of mouse GAPDH were amplified as markers of nuclear and

cytoplasmic RNA, respectively. The data represent the average ± SEM of three separate experiments. (**E**) Overexpression of miR-29b and miR-18b downregulated the mRNA levels of Col4a1 and CTGF in EWS WT MEFs. The mRNAs were detected by qRT-PCR. The data represent the average ± SEM of five separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$. (**F**) Overexpression of miR-29b and miR-18b reduced the protein level of Col4a1 and CTGF in EWS WT MEFs.

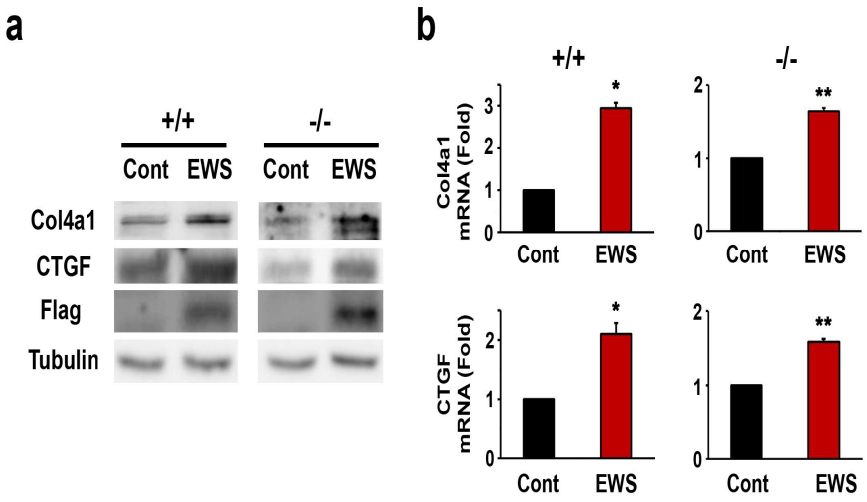

Figure 5. Overexpression of EWS leads to increases of Col4a1 and CTGF expression.

(A) Overexpression of EWS increased the protein levels of Col4a1 and CTGF were in EWS MEFs. (B) qRT-PCR analysis represented that overexpression of EWS incrased the mRNA levels of Col4a1 and CTGF in EWS MEF cells. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$.

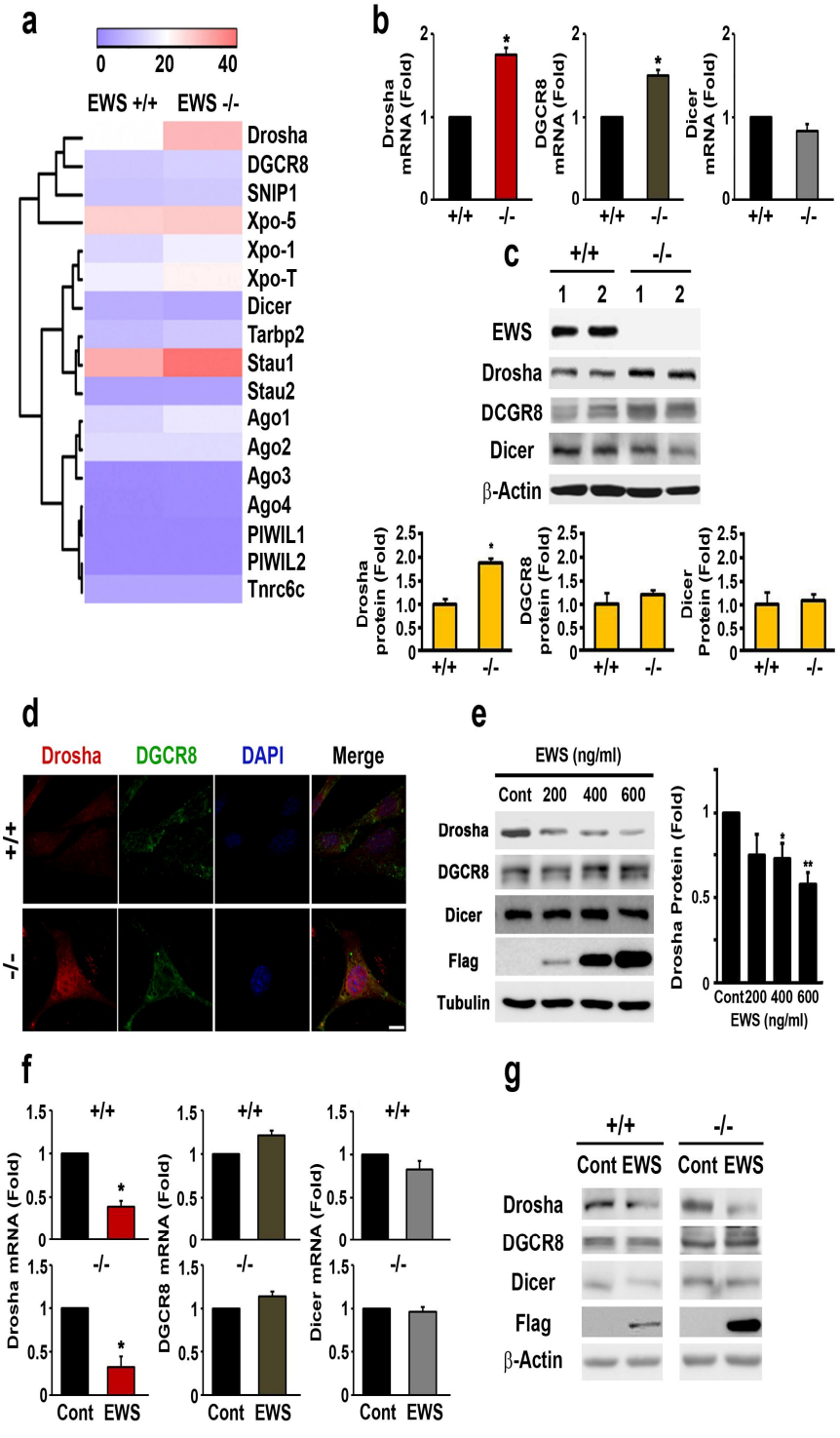
a
0
20
40
EWS +/+
EWS -/-
Drosha
DGCR8
SNIP1
Xpo-5
Xpo-1
Xpo-T
Dicer
Tarbp2
Stau1
Stau2
Ago1
Ago2
Ago3
Ago4
PIWIL1
PIWIL2
Tnrc6c
b
Drosha mRNA (Fold)
DGCR8 mRNA (Fold)
Dicer mRNA (Fold)
+/+
-/-
c
+/+
-/-
1
2
1
2
EWS
Drosha
DCGR8
Dicer
β-Actin
Drosha protein (Fold)
DGCR8 protein (Fold)
Dicer Protein (Fold)
d
Drosha
DGCR8
DAPI
Merge
+/+
-/-
e
EWS (ng/ml)
Cont
200
400
600
Drosha
DGCR8
Dicer
Flag
Tubulin
Drosha Protein (Fold)
Cont200 400 600
EWS (ng/ml)
f
+/+
-/-
Drosha mRNA (Fold)
DGCR8 mRNA (Fold)
Dicer mRNA (Fold)
Cont
EWS
g
+/+
-/-
Cont EWS
Cont EWS
Drosha
DGCR8
Dicer
Flag
β-Actin

Figure 6. EWS regulates Drosha expression.

(A) The heat map of transcriptome analysis showed relative expressions of miRNA processing-related genes in between EWS WT and KO MEFs. *Drosha* among many microprocessors was increased in EWS KO cells while other genes were barely changed. The relative expression level of genes was displayed as colors: higher (pale red) or lower (violet). (B) qRT-PCR verified that the mRNA level of Drosha was most significantly increased in EWS KO MEF. The data represent the average ± SEM of three separate experiments. (C) Western blot analysis confirmed that the protein level of Drosha elevated in EWS KO MEF. Two independent experiments were performed for Western blot analysis. The graphs represent the average ± SEM of three separate experiments. (D) The confocal microscopy presented that the immunoreactivity of Drosha is highly increased in the nucleus of EWS KO MEF while the immunoreactivity of DGCR8 is not changed. Scale bar: 10μm. (E) Overexpression of EWS reduced the protein level of Drosha in a dose dependent manner. The graph represents the average ± SEM of five separate experiments (F) Overexpression of EWS decreased the mRNA level of Drosha both in EWS WT and

KO MEFs. The data represent the average ± SEM of five separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$. (G) Overexpression of EWS reduced the protein levels of Drosha both in EWS WT and KO MEFs.

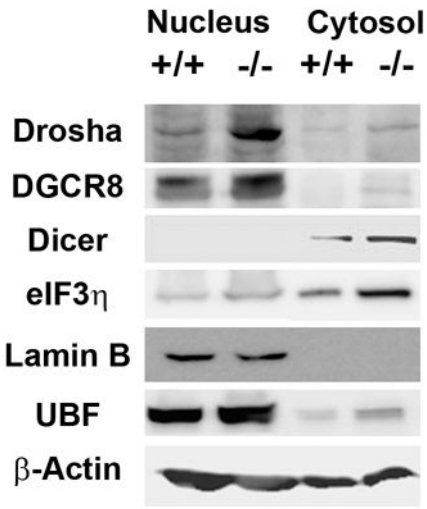

Figure 7. Drosha is elevated in the nucleus of EWS KO MEF cells.

Subcellular fractionation analysis showed that Drosha is mainly increased in the nucleus of EWS KO (−/−) cells. The protein levels of DGCR8 and Dicer were not changed. Lamin B, nucleus marker; UBF, nucleolar marker; and eIF3η, cytosolic marker.

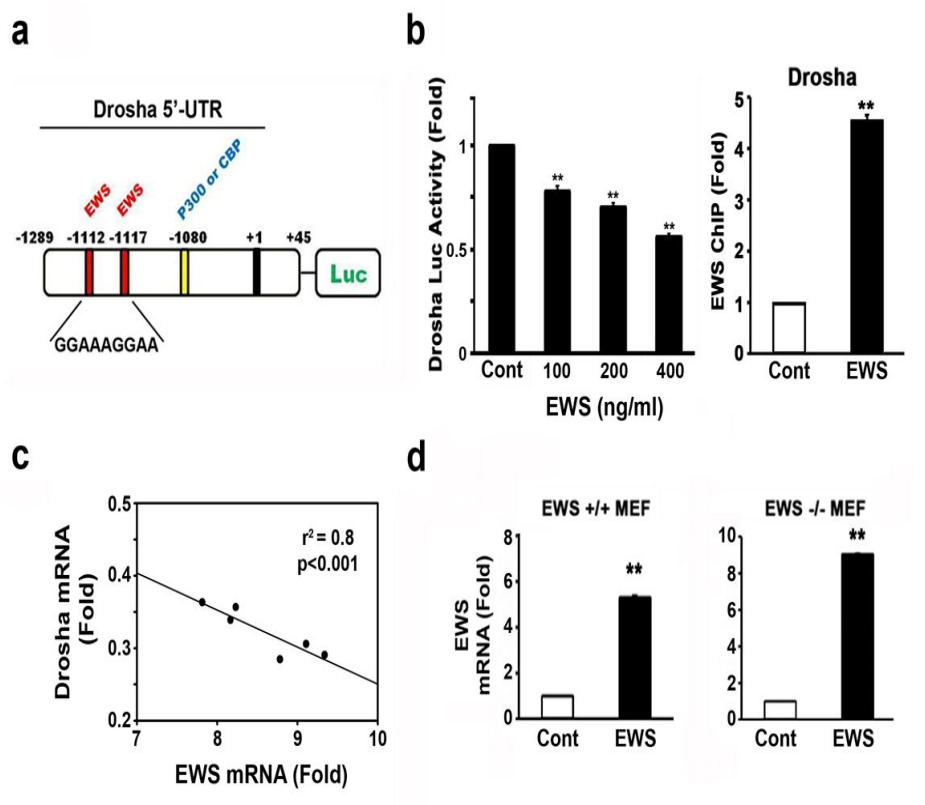

Figure 8. EWS directly regulates the expression of Drosha.

(A) The 5'-UTR of mouse Drosha promoter shows putative EWS and CBP binding elements. The 5'-UTR of mouse Drosha was cloned into pGL4.14 luciferase vector. (B) EWS down regulated the transcriptional activity of Drosha promoter in a dose dependent manner in NIH 3T3 cells. The EWS-DNA occupancy within the mouse Drosha promoter region was increased in NIH 3T3 cells. The data represents the average ± SEM of three separate

experiments. (C) The expression level of Drosha mRNA is inversely correlated with the level of EWS. The regression analysis was derived from the Figure 4G and the Supplementary Figure S4D. (D) The ectopic expression of EWS was determined by qRT-PCR after transfection EWS in EWS MEF cells. The data represent the average ± SEM of three separate experiments. Significantly different at **, $p<0.005$.

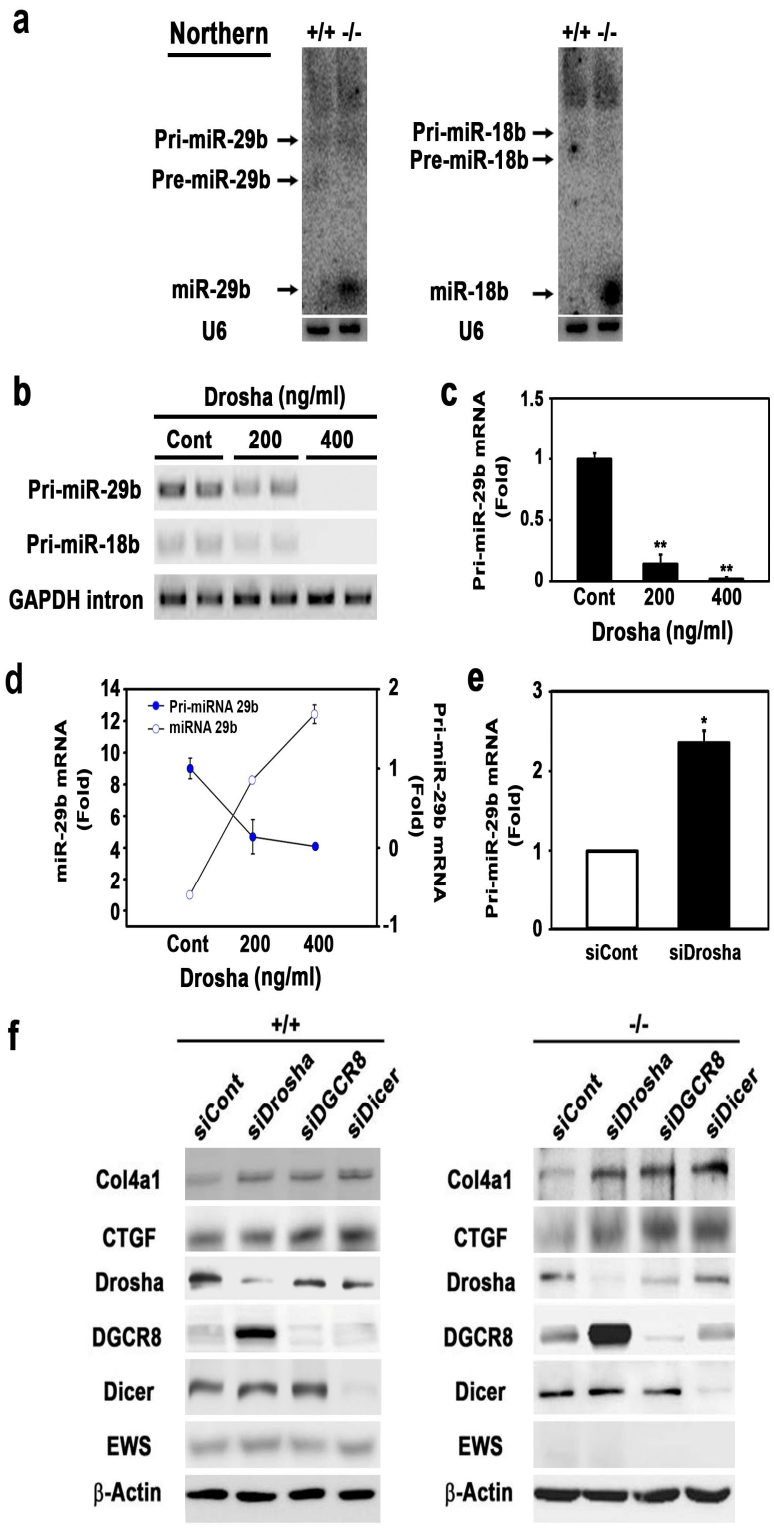
a
Northern
+/+ -/-
Pri-miR-29b
Pre-miR-29b
miR-29b
U6
+/+ -/-
Pri-miR-18b
Pre-miR-18b
miR-18b
U6
b
Drosha (ng/ml)
Cont
200
400
Pri-miR-29b
Pri-miR-18b
GAPDH intron
c
Pri-miR-29b mRNA (Fold)
1.5
1
0.5
0
**
**
Cont
200
400
Drosha (ng/ml)
d
Pri-miRNA 29b
miRNA 29b
miR-29b mRNA (Fold)
Pri-miR-29b mRNA (Fold)
14
12
10
8
6
4
2
0
2
1
0
-1
Cont
200
400
Drosha (ng/ml)
e
Pri-miR-29b mRNA (Fold)
3
2
1
0
*
siCont
siDrosha
f
+/+
-/-
siCont
siDrosha
siDGCR8
siDicer
Col4a1
CTGF
Drosha
DGCR8
Dicer
EWS
β-Actin

Figure 9. Drosha regulates the processing of pri-miR-29b and pri-miR-18b and involves in Col4a1 and CTGF expression.

(**A**) Northern blot analysis of miR-29b and miR-18b in EWS MEF cells. Mature forms of the miR-29b and miR-18b were highly expressed in EWS KO MEFs. U6 was used as a loading control. (**B**) RT-PCR analysis revealed that the levels of pri-miR-29b and pri-miR-18b are decreased by Drosha in a dose dependent manner (**C**) qRT-PCR analysis verified that Drosha reduces the level of pri-miR-29b in dose dependent manner. The data represent the average ± SEM of three separate experiments. (**D**) Drosha increased the level of miR-29b in dose dependent manner. The data represent the average ± SEM of three separate experiments. (**E**) Knock down of Drosha increased the level of pri-miR-29b. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$. (**F**) Knock down of Drosha, DGCR8, and Dicer

increased Col4a1 and CTGF protein levels both in EWS WT and KO MEFs.

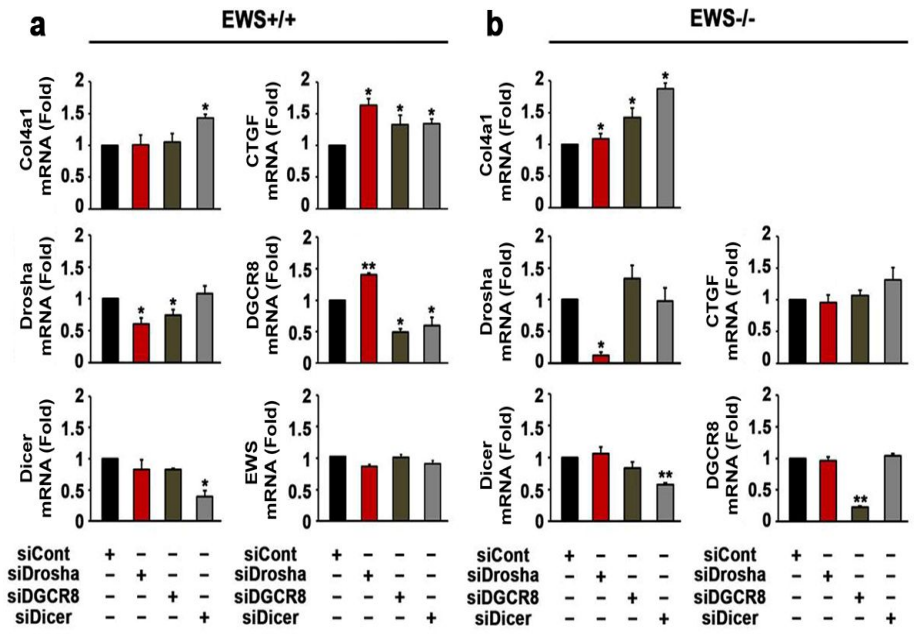

Figure 10. microRNAs are involved in Col4a1 and CTGF expression.

(A) Knock down of Drosha, DGCR8, and Dicer elevated Col4a1 and CTGF mRNA levels in EWS WT MEFs. (B) Knock down of Drosha, DGCR8, and Dicer increased Col4a1 and CTGF mRNA levels in EWS KO MEFs. siDrosha, siDGCR8, and siDicer were transfected in EWS WT and KO MEFs. The mRNA levels were normalized to GAPDH. The data represent the average ± SEM of five separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$.

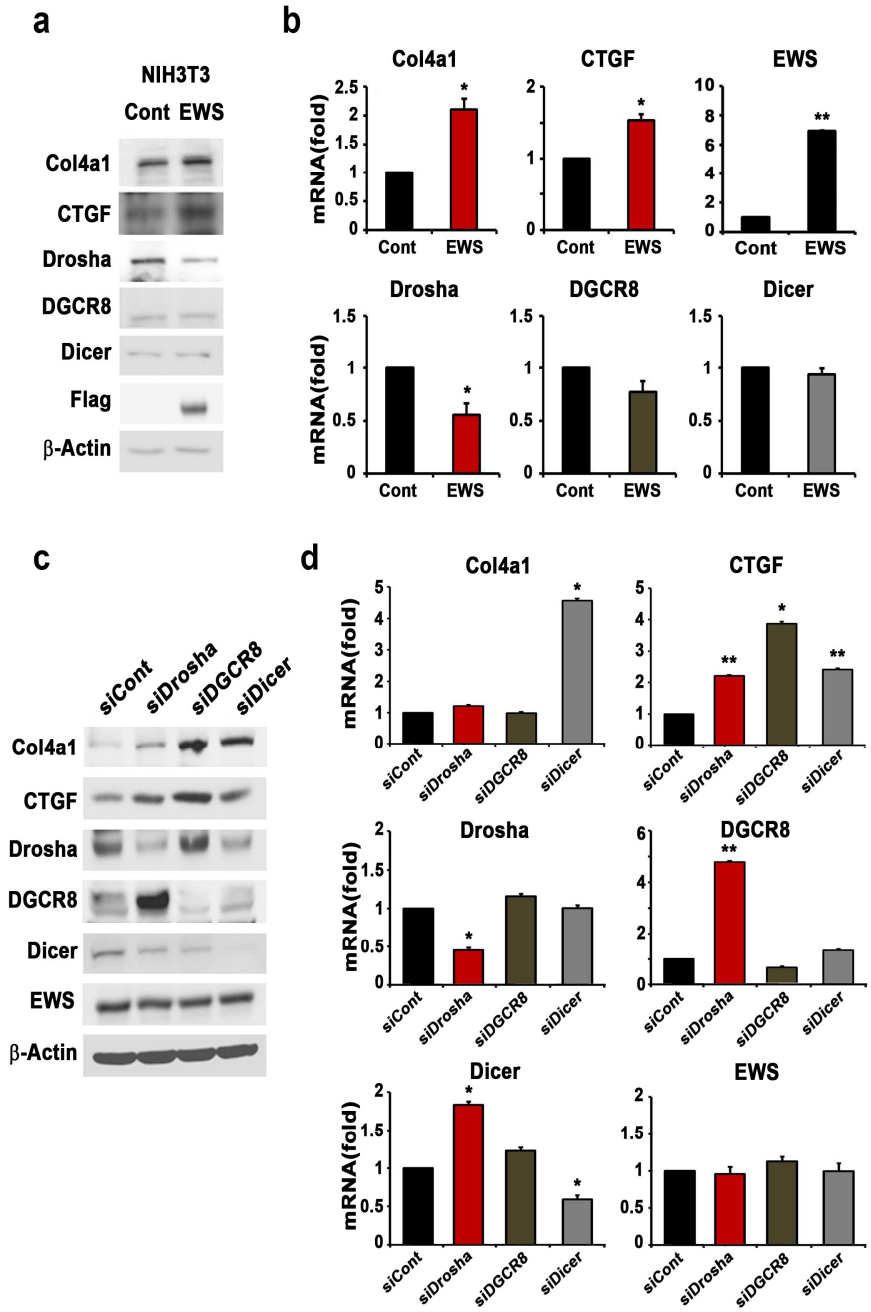

Figure 11. EWS and microprocessors involve in the posttranscriptional regulation of Col4a1 and CTGF in NIH 3T3 cells.

(A)Overexpression of EWS significantly increased the protein levels of Col4a1 and CTGF but decreased the protein level of Drosha. The protein level of DGCR8 and Dicer was barely changed. (B) Overexpression of EWS increases the mRNA levels of Col4a1 and CTGF but decreased the mRNA level of Drosha. The data represent the average ± SEM of three separate experiments. (C) Knock down of Drosha, DGCR8, and Dicer increased the protein level of Col4a1 and CTGF. (D) Knock down of Drosha, DGCR8, and Dicer significantly increased the mRNA levels of Col4a1. The data represent the average ± SEM of five separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$.

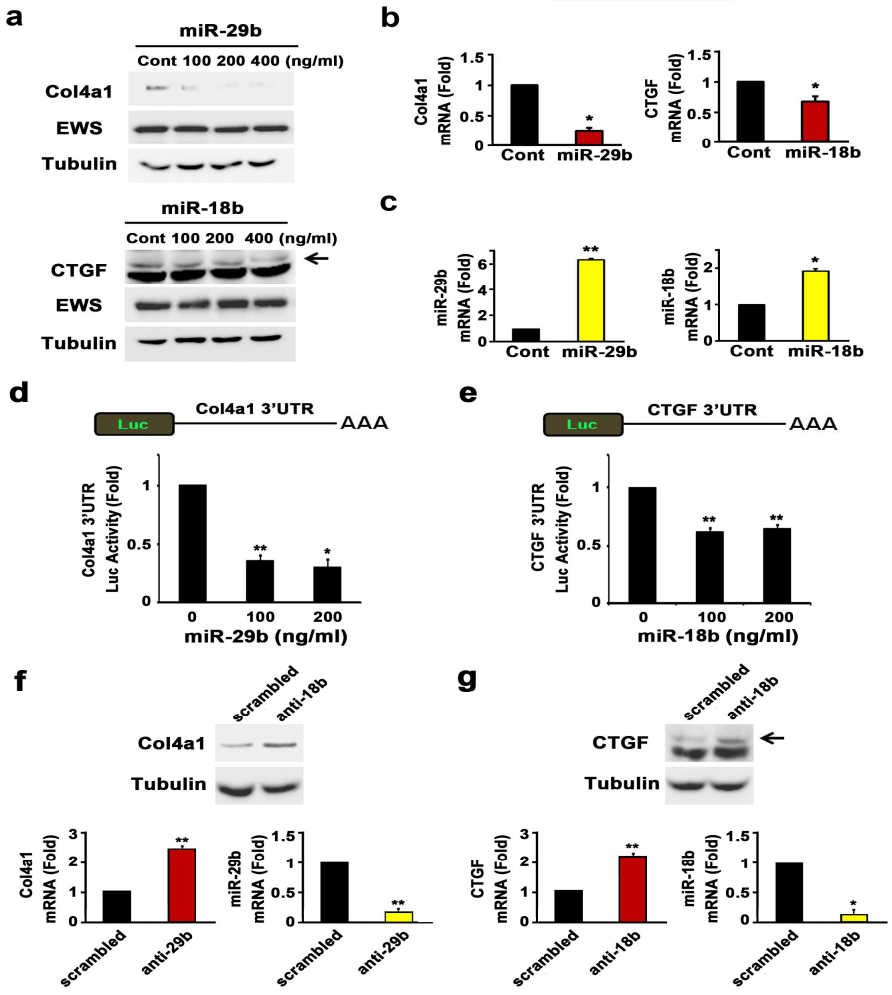

Figure 12. miR-29b and miR-18b negatively regulates Col4a1 and CTGF in NIH 3T3 cells.

(A)Overexpression of miR-29b and miR-18b significantly decreased the protein levels of Col4a1 and CTGF in a dose dependent manner, respectively. (B) Overexpression of miR-

29b and miR-18b significantly decreased the mRNA levels of Col4a1 and CTGF. The data represent the average ± SEM of three separate experiments. (C) qRT-PCR analysis of miR-29b and miR-18b confirmed the ectopically expressed levels of miR-29b and miR-18b in NIH 3T3 cells. The mRNA levels of miRNAs were normalized to 5S RNA. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$. (D) The 3'UTR of mouse Col4a1 was cloned into pmirGLO dual-luciferase vector. miR-29b down regulated the transcriptional activity of Col4a1 3'UTR in a dose dependent manner in NIH 3T3 cells. (E) The 3'UTR of mouse CTGF was cloned into pmirGLO dual-luciferase vector. miR-18b down regulated the transcriptional activity of CTGF 3'UTR in a dose dependent manner in NIH 3T3 cells. The data represent the average ± SEM of three separate experiments. (F) Transfection of miR-29b inhibitor (anti-29b) increased col4a1 protein and mRNA levels and decreased miR-29b in NIH 3T3 cells. (G) Transfection of miR-18b inhibitor (anti-18b) increased CTGF protein and mRNA levels and decreased miR-18b in NIH 3T3 cells. The data represent the average ± SEM of three separate experiments. The mRNA

levels of miRNAs were normalized to 5S RNA. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$.

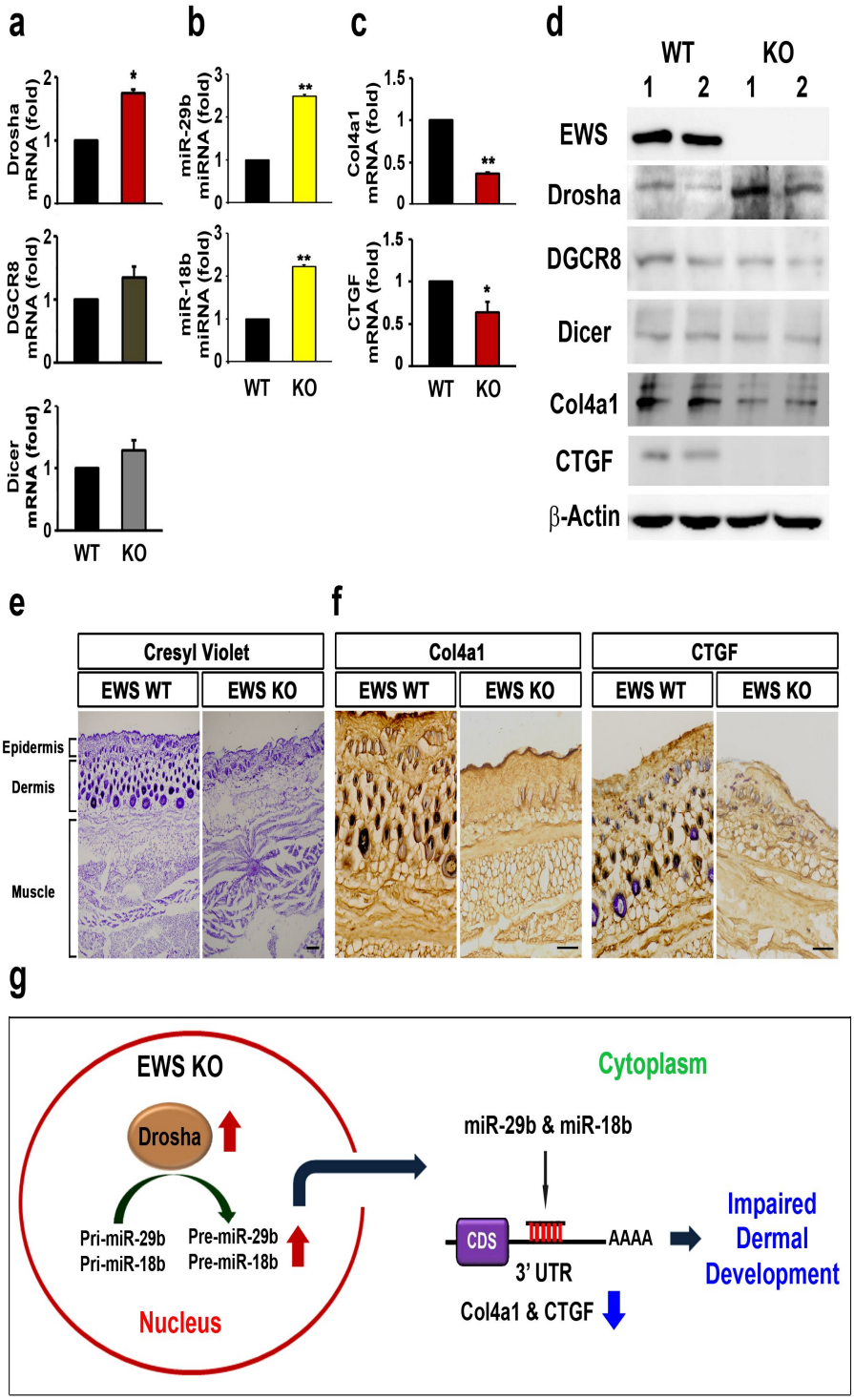
a
b
c
d
Drosha mRNA (fold)
DGCR8 mRNA (fold)
Dicer mRNA (fold)
miR-29b miRNA (fold)
miR-18b miRNA (fold)
Col4a1 mRNA (fold)
CTGF mRNA (fold)
WT
KO
WT KO
1 2 1 2
EWS
Drosha
DGCR8
Dicer
Col4a1
CTGF
β-Actin
e
f
Cresyl Violet
Col4a1
CTGF
EWS WT
EWS KO
Epidermis
Dermis
Muscle
g
EWS KO
Drosha
Pri-miR-29b
Pri-miR-18b
Pre-miR-29b
Pre-miR-18b
Nucleus
Cytoplasm
miR-29b & miR-18b
CDS
AAAA
3' UTR
Col4a1 & CTGF
Impaired
Dermal
Development

Figure 13. EWS KO mice show the alteration of Drosha, miRNAs and Col4a1 and CTGF.

(A) Drosha mRNA levels were increased in EWS KO mice. The data represent the average ± SEM of three separate experiments. (B) miR-29b and miR-18b levels were markedly increased in EWS KO mice. (C) Col4a1 and CTGF mRNA levels were reduced in EWS KO mice. The data represent the average ± SEM of three separate experiments. Significantly different at *, $p<0.05$; **, $p<0.005$. (D) The protein levels of Drosha were increased in EWS KO mice whereas the protein levels of Col4a1 and CTGF were downregulated in the skin tissues of EWS KO mice (n=2). (E) Nissl staining showed severely altered skin structure of EWS KO mice. (F) Col4a1 and CTGF immunoreactivity were decreased in EWS KO mice skin tissues. All scale bars: 100 μm. (G) A schematic diagram illustrates that the upregulation of Drosha under EWS deficiency accelerates the processing of pri-miR-29b and pri-miR-18b in the nucleus. Increased miR-29b and miR-18b negatively regulate the mRNAs of Col4a1 and CTGF in the cytoplasm. As a result, EWS deficiency leads to impaired dermal development.

Table 1 miRNAs and their target genes in EWS KO cells.

miRNA	Target
miR-29b	Col4a1 (collagen, type IV, alpha 1)
miR-18b	CTGF (connective tissue growth factor)
miR-130b	DMPK (dystrophia myotonica-protein kinase)
miR-103 and miR-501-5p	Myom1 (myomesin 1)

miRNA array revealed that miR-29b, miR-18b, miR-130b, miR-103, and miR-501-5p are highly increased in EWS KO cells.

Table 2 Primer sequences that are used for quantitative real-time PCR (qRT-PCR).

Gene	Sequence
Col4a1	F- AAA AGG ACA GCA AGG TGT GA R- CTA TGC CTG GTC ACA AGG AA
Col8a1	F- TAT GGC AAA GAG TAC CCA CA R- CTT TGA TTC CAG GCA TTC CA
CTGF	F- GTG TGA AGA CAT ACA GGG CTA R- TTC CTG TAG TAC AGG GAC TCA
DMPK	F- GTC CTG CTG AGA TAA GGC TA R- ATG TCT TCC TGC ATG TCT GA
FGF10	F- CCT GGA GAT AAC ATC AGT GGA R- GCT GCC AGT TAA AAG ATG CA
FGFR2	F- TAA CAC CAC GGA CAA AGA GA R- GGC GAT TAA GAA GAC CCC TA
MGP	F- CTG TGC TAC GAA TCT CAC GA R- GCT CAC ACA GCT TGT AGT CA
MYOM1	F- TTG TTC GAG ATG CTG ATG CA R- GAA ACC GAG CAA ACT TCA CA
ASPN	F- TAT CAG GAT CGC TGA AGC AA R- GTA TCT CTC TCA CAC GTG GTA
ENG	F- GGA ATG CTG TCA CAT CTG GA R- CCT GGA GGT AAG GGA TGG TA
PRRX1	F- CAG GAC AAC ATT CAA CAG CA R- TCC TGA GTA GGA CTT GAG GA
Serpinb1a	F- ACA GAA GGG AAA ATC CCA GA R- CAG GTC CGA AAT GTA ACC AA
Drosha	F- TTC GCT GTA CAC ATT CGG AA R- CTC ACA TTC AGA CTC ACT GGA
DGCR8	F- CTT ACG GAT CTG GAA CTG CA R- CAT GGA GGA TCT GAT ATG GAG A
Dicer	F- CTA GAC CAC CCC TAT CGT GA R- CCA CAA TCT CAC AAG GCT GA
EWS	F- GTT ATA ACC AAC CCA GCC TA R- GAG TAA CTG CTC TGA TCG TA
GAPDH	F- TGT GTC CGT CGT GGA TCT GA R- CCT GCT TCA CCA CCT TCT TGA

Table 3 Primer sequences that are used for reverse transcriptase-PCR (RT-PCR).

Gene	Sequence
Col4a1 intron 1	F- GAC TCT GCT CCT CTG GTC AC R- GAG GTC CTC GGT CTC CTT TG
Col4a1 intron 2	F- AGA TGT TTG CAA GAG CCA GA R- AAA CTT GGA AGC TTC GGT GA
Col4a1 exon	F- AAA AGG ACA GCA AGG TGT GA R- CTA TGC CTG GTC ACA AGG AA
CTGF intron 1	F- GGC TAG AAT GAG AGA TGC TGT R- GCA ATT ACT ACA ACG GGA GTG
CTGF	F- GTG TGA AGA CAT ACA GGG CTA R- TTC CTG TAG TAC AGG GAC TCA
GAPDH intron	F- CTG AGT CAT GGT GGT TCT GA R- GGA GAG ATC TGG TTT CTG GA
GAPDH exon	F- TGA CAT CAA GAA GGT GGT GAA GCA G R- GGT CCA CCA CCC TGT TGC TGT AG
Pri-miR-29b	F- AAC TAT TGC ACG GAC TTC AC R- ACA CTG GAC ACT TAC TTC AG
Pri-miR-18b	F- ACC ATG GTG ATT TAA TCA GA R- CCG TTC AAA TCA TTT CTC AA

DISCUSSION

EWS is a member of the TET family of proteins (TLS/FUS, EWS and TAF15) and is found frequently fused to several transcription factors containing different DNA-binding domains(3,8). EWS fusion proteins, such as FLI1,(1) or EWS interaction partners, such as CREB binding protein (CBP),(3) can control the transcription of mRNA of many genes. It has been previously demonstrated that EWS is part of the Drosha/DGCR complex, but the potential roles for EWS in miRNA biogenesis or regulation has not been examined. In the present study, we found that several miRNAs are altered as a result of EWS inactivation. Among them, we discovered that miR-29b and miR-18b are significantly upregulated in the absence of EWS and subsequently, expression of Col4a1 and CTGF were decreased in EWS KO cells. Interestingly, we found that Drosha, one of the most important miRNAs processing molecules, was significantly elevated in EWS KO cells as seen on RNA sequencing analysis, by showing the inverse correlation with the levels of Col4a1 and CTGF. This data led

us to hypothesize that the processing of miRNA might be affected in a Drosha-dependent manner and that altered miRNAs may participate in the posttranscriptional regulation of Col41 and CTGF under EWS deficiency.

Drosha is a miRNA processor and is essential for miRNA maturation. Drosha interacts with DGCR8 to process pri-miRNA to pre-miRNA (21). Both Drosha and DGCR8 proteins are presented abundantly and ubiquitously but the expression level of these proteins depends on the cell types (22). If Drosha levels are elevated in the cell, more pre-miRNAs will be produced and, consequently, more miRNAs will be processed in a cell-specific manner. This pre-miRNAs processing is dependent of EWS which is a transcription factor. In this paradigm, our study indicates that Drosha is directly regulated by EWS as an upstream target of the miRNA processing pathway. Drosha promoter assay showed that the transcription of Drosha is downregulated by EWS in a dose dependent manner because of that we found EWS and CBP binding sites on the Drosha promoter region. Our EWS ChIP data showing that EWS-DNA occupancy is highly increased within the 5'-UTR promoter region of Drosha, supports a role

of EWS in the regulation of Drosha transcription. Since Drosha is upregulated in EWS KO cells, it is expected that the processing of pri-miRNA to pre-miRNA could subsequently be facilitated (23). Dicer then converts the pre-miRNA into a small RNA duplex, which combines with the RNA induced silencing complex (RISC) (22). Mature miRNA processing by Dicer would be changed because Drosha expression is down regulated in EWS deficiency condition, but according to the recent reports pre-miRNA processing by over expressed Drosha does effect on mature miRNAs processing (15) because the Dicer processing ability is still now known.

As predicted by the increased Drosha expression in EWS KO cells, pri-miRNAs for miR-29b and miR-18b are markedly decreased in EWS KO cells while the two miRNAs were highly increased in EWS KO cells. The increases in the level of miR-29b and miR-18b were correlated with post-transcriptional regulation of Col4a1 and CTGF in the cytoplasm of EWS KO cells. Overexpression of miR-29b and miR-18b significantly down regulated the mRNA of Col4a1 and CTGF, respectively, in a dose dependent manner. In contrast, miR-29b inhibitor (anti-29b) and miR-18b inhibitor (anti-18b) up

regulated the protein and mRNA levels of Col4a1 and CTGF. It is apparent that miR-29b and miR-18b induce the degradation of target mRNAs act as a major mode of action (17). Importantly the levels of miR-29b and miR-18b were significantly elevated in the skin tissues of EWS KO mice. In addition, both the mRNA and the protein levels of Drosha were elevated in the skin tissues of EWS KO mice while the proteins levels of DGCR8 and Dicer were slightly changed. In contrast, the mRNA and protein levels of Col4a1 and CTGF were significantly reduced in the skin tissues of EWS KO mice. These results indicate that Col4a1 and CTGF are posttranscriptionally regulated by Drosha-dependent miRNA pathway under EWS deficiency *in vivo*. Previous studies have shown that Col4a1 and CTGF play a critical role in the dermal development (9,14). miR-18/19 plays a role in the fine-tuning of extracellular matrix (ECM) protein levels during the aging of cardiomyocytes (20). An inverse correlation between the level of miR-18/19 and the level of CTGF clearly shows that small noncoding microRNAs regulate gene expression at the post-transcriptional level. Thus, the changes of specific miRNA and its-targeted ECM protein are implicated in the aging process

(20). Otherwise, the effects of miR-29b on the expression of collagen genes that are related to the constitution of extracellular matrix and the possible protective role of miR-29b in the renal injury have been examined (17). A two fold increase in miR-29b levels is associated with reciprocal expression of Col4a1 in bleomycin-induced pulmonary fibrosis (29). This suggests that this level of increase in EWS KO MEFs could have a physiologically significant effect. However, the direct regulatory mechanism of these miRNAs by microprocessor complex and its role in the skin development has not been determined. For the first time, our study present that Drosha-dependent miRNA processing of the miR-18b and miR-29b is directly associated with a failure of skin development in EWS KO mice. Indeed, knock-down of Drosha, DGCR8 and Dicer by siRNAs recovered the mRNA and protein levels of Col4a1 and CTGF. In this context, EWS modulation of Drosha and miRNAs may enhance the expression of Col4a1 and CTGF to improve dermal morphogenesis (18).

In summary, we provide evidence that EWS modulates the expression of Col4a1 and CTGF by regulating miRNA-29b and miRNA-18b (Figure 13G). Furthermore, increased Drosha

expression mediates the processing of pre-miRNAs, promoting the biogenesis of miR-29b and miR-18b, and subsequently reducing post-transcription levels of Col4a1 and CTGF under EWS deficiency. We found that EWS, a transcription factor with CBP/p300, plays a permissive role in the upregulation of Drosha and miRNA processing. The exact nature by which EWS regulates Drosha expression and the microprocessor complex needs to be further examined. Identifying the targets of other miRNAs that are altered as a result of EWS inactivation will reveal additional roles for the multifunctional EWS in different and as yet undescribed cellular processes.

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국문 초록

Ewing's Sarcoma 에서 유래된 EWS 는 발암성 유전자이며 이는 RNA/DNA 에 결합하는 것으로 전사조절 기능을 가지며 세포내의 여러 과정들에 관여하며 세포 내 존재한다. EWS 의 결핍은 세포 발달과 초기 노화에 관계하며 이들 과정의 원인은 아직 밝혀지지 않았다. 본 연구에서는 Drosha and 마이크로 RNA 가 CBP/p300 과의 상호작용으로 인한 전사 조절에 관여하는 EWS 단백질에 의해서 발현이 조절되어짐을 밝히고 확인하였다. Drosha 는 마이크로 RNA 생성에 있어서 중요한 요소이며 이는 EWS 단백질의 결핍 시 증가한다 그 이유는 Drosha 의 5'-UTR 부분에 EWS 와 CBP/p300 의 결합부위가 있으므로 EWS 와 CBP/p300 의 상호작용으로 Drosha 의 전사 억제되는 것을 확인하였다. 이러한 작용으로 마이크로 RNA-29b 와 마이크로 RNA-18b 또한 증가하는 것을 확인하였다. EWS knock-out 쥐의 배아 섬유아세포에서 증가한 마이크로 RNA-29b 와 마이크로 RNA-18b 는 특이적으로 Col4a1 과 CTGF 를 각각 전사 후 과정에서 조절하는 것을 확인하였다. EWS 결핍 시 EWS 와 CBP/p300 의 상화 작용으로 인하여 Drosha 의 메신저 RNA 의 전사억제의 기능을 저해 하므로 증가하는 Drosha 와 EWS 에 의해서 증가된 pri-마이크로 RNA-

29b 와 pri-마이크로 RNA-18b 는 마이크로 RNA-29b 와 마이크로 RNA-18 의 생성을 증가시키며 이는 Col4a1 과 CTGF 의 메신저 RNA 를 조절함으로써 EWS KO 쥐의 피부 발달을 저해함을 확인하였다. EWS 를 인위적으로 과 발현 시 Drosha 와 마이크로 RNA-29b 와 마이크로 RNA-18b 는 감소함을 확인하였고 Col4a1 과 CTGF 는 증가함을 확인하였다. Dicer 는 마이크로 RNA 생성을 최종적으로 하게 되는데 이는 EWS 가 발현되지 않는 조건에서 Drosha 의 변화와 마이크로 RNA 의 생성에 관여 할 수 있음을 알 수 있다 하지만 최근 보고된 여러 논문들의 결과에서는 Drosha 의 증가는 Dicer 의 변화와 관계없이 마이크로 RNA 의 생성을 증가시킨다 그 이유는 Dcier 의 마이크로 RNA 생성능력이 정확히 알려지지 않았기 때문이다. 결과적으로, EWS 는 Drosha 와 마이크로 RNA-29b 와 마이크로 RNA-18b 발현양을 조절하며 이들 마이크로 RNA 는 Col4a1 과 CTGF 의 메신저 RNA 를 조절함으로써 피부 형성에 새로운 메커니즘을 제시한다.

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