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E2F1-regulated DROSHA promotes miR-630 biosynthesis in cisplatin-exposed cancer cells

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ABSTRACT

DNA damage may regulate microRNA (miRNA) biosynthesis at the levels of miRNA transcription, processing and maturation. Although involvement of E2F1 in the regulation of miRNA gene activation in response to DNA damage has been documented, little is known about the role of E2F1 in miRNA processing. In this study we demonstrate that E2F1 enhances miR-630 biosynthesis under cisplatin (CIS) exposure through promoting DROSHA-mediated pri-miR-630 processing. Northern blot and RT-qPCR revealed that CIS exposure caused not only an increase in pri-miR-630 but also much more increase in pre-miR-630 and mature miR-630. The increases in pri-miR-630 and pre-miR-630 expression in unmatched proportion indicated that primary transcript processing was involved in CIS-stimulated miR-630 biosynthesis. Furthermore, combination of reporter enzyme assay with mutation and over-expression of E2F1 showed that induction of DROSHA promoted miR-630 expression, in which CIS-induced E2F1 activated *DROSHA* gene expression by recognizing and binding two E2F1 sites at the positions −214/−207 and −167/−160 of the DROSHA promoter. The increased binding of E2F1 to the DROSHA promoter in CIS-exposed cells was further evidenced by chromatin immunoprecipitation assay. Together, E2F1-regulated DROSHA promotes pri-miR-630 processing, thereby, contributes to CIS-stimulated miR-630 expression. The involvement of E2F1-dependent DROSHA activation in pri-miRNA processing under DNA damage stress will provide further insight into the regulation of miRNA biosynthesis. These data also give us a deeper understanding of E2F1 role in response to DNA damage.

1. Introduction

miRNAs are a class of small non-coding RNAs, regulating gene expression at post-transcription levels through either inhibiting target mRNA translation or inducing its degradation [1,2]. The majority of miRNA genes are transcribed by RNA-polymerase II (RNA Pol II) [2,3] except to a miRNA cluster that is located between *Alu* repeats on chromosome 19 and transcribed by RNA-polymerase III [4]. The generation of miRNAs is a multistep process. Briefly, miRNA genes are transcribed by RNA Pol II into primary miRNA transcripts (pri-miRNA) in the nucleus [2,3], and then processed by DROSHA-DGCR8 complex to generate ~70 nt stem-loop precursor miRNA (pre-miRNA) [5,6]. These pre-miRNAs are exported from the nucleus to the cytoplasm and processed into 22 nt mature miRNA duplexes by the RNase III DICER enzyme [7,8]. Finally, the mature single–stranded miRNAs assemble with argonaute proteins into RNA-inducing silencing complex (RISC) and direct the RISC complex to target mRNA [9].

MiRNAs play crucial roles in diverse biological processes such as cell proliferation, differentiation, and apoptosis [10–12]. Furthermore, miRNAs have been shown to be involved in response to DNA damage [12,13]. For instance, miRNA-34 family members are regulated by p53 upon DNA damage and play roles in cell-cycle checkpoint and apoptosis [11]. In addition, certain miRNAs are involved in DNA damage and repair [14]. It is believed that regulation of DNA damage response by miRNAs may have the potential to improve the efficacy of cancer chemotherapy and radiotherapy relying on the induction of DNA damage [12,13]. Further understanding of miRNA biogenesis and actions in response to DNA damage will provide insights into tumorigenesis and limiting tumor progression.

Transcription factor E2F1 plays roles in the regulation of cell-cycle progression through regulating a very diverse array of genes [15,16]. In addition, E2F1 as a DNA damage responsive transcription factor also regulates genes responsible for checkpoint activation, DNA repair, and apoptosis, participating in maintaining genomic integrity under DNA damage stress [15,16]. The emerging evidences have demonstrated that E2F1 can directly activate miRNA gene expression [17,18]. However, little is known about the role

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of E2F1 in miRNA processing. We have previously shown that E2F1 is induced by DNA damage [19,20]. Since we found recently that miR-630 as well as E2F1 was induced upon cisplatin (CIS) induced DNA damage, we analyzed the consensus E2F site within the sequence of human DROSHA promoter and found two putative E2F binding sites in the promoter. In this study, we demonstrate that the induction of miR-630 upon CIS exposure is attributed at least partly to E2F1-dependent activation of DROSHA that promotes miRNA processing. Our data will strengthen our understanding of anticancer chemotherapeutics. Also, E2F1-dependent DROSHA activation in miRNA processing will provide further insight into the regulation of miRNA biosynthesis.

2. Materials and methods

2.1. Cell culture and drug treatment

A549 and H1299 cells were maintained in DMEM medium with 10% fetal bovine serum (GIBCO BRL). Cells were incubated in an atmosphere of 5% CO_2 at 37 °C. Cells were exposed to cisplatin (Sigma) for given hours.

2.2. Constructs, transfection and RNA interference

pcDNA3-E2F1 was kindly provided by Dr. Joseph R. Nevins. For construction of luciferase reporter plasmids, the DROSHA promoter extending from −893 to +142 was amplified using A549 genomic DNA as template and inserted to pGL3 to generate pGL3-DROSHA (−893/+142) reporter construct. 5′ deletion (Fig. 3F) and substitution (Fig. 3G) mutants were constructed by PCR using pGL3-DROSHA (−893/+142) as template. The reverse primer for all PCR amplification was 5′-CCGCTCGAGCAGGCTACTACCGCAG GT-3′; forward primers for −893/+142: 5′-CTAGCTAGCACAGAAGA ATCGCTCGAA-3′; −433/+142: 5′-CGACGCGTACAGCAACGGAATAG GGC-3′; −292/+142: 5′-CGACGCGTTGATGATCCTCCACCACCC-3′; −198/+142: 5′-CGACGCGTTTGCAGTAAAGCGGTGGC-3′; −100/ +142: 5′-CTAGCTAGCTTTTCGGATTGGAGGACG-3′.

MuE2F1 for binding site 1:

F: 5′-CGCGGAGTCACTCATGTTTTCAAGCCCGTCAGTTTTGCAG-3′
R: 5′-CTGCAAAACTGACGGGCTTGAAAACATGAGTGACTCCGCG-3′

MuE2F1 for binding site 2:

F: 5′-AGCTGGAGAAGACCCCAATAAGCCGGCGCCTCATCCCGGC-3′
R: 5′-GCCGGGATGAGGCGCCGGCTTATTGGGGTCTTCTCCAGCT-3′.

Chemically synthesized siRNAs were from GenePharma (Shanghai, China). Transfection was performed with Lipofectamine 2000 (Invitrogen), following manufacturer's instructions.

2.3. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using Trizol Reagent (Invitrogen). For mature miRNA detecting, cDNA synthesis was performed by RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific) with miRNA-specific primers. For pri-miRNA and mRNA quantity, total RNA was reversely transcribed with random primer using RevertAid™ First Strand cDNA Synthesis Kit. Power SYBR Green PCR Master Mix (Applied Biosystems) was used for real-time PCR applications. Primers were as follows: pri-miR-630, 5′-TAAAGGAG-GAAGATAAGG-3′ and 5′-GTAGCAGTGATAGGCATT-3′; DROSHA, 5′-TAGGCTGTGGGAAAGGACCAAG-3′ and 5′-GTTCGATGAACCGCTT CTGATG-3′; GAPDH, 5′-TGTCAGTGGTGGACCTGACCT-3′ and 5′-AG GGGAGATTCAGTGTGGTG-3′; miR-630, 5′-ACACTCCAGCTGGGAGT ATTCTGTACCAG-3′ and 5′-TGGTGTCGTGGAGTCG-3′; U6, 5′-CCTGC TTCGGCAGCACA-3′ and 5′-TGGAACGCTTCACGAA-3′; Stem-loop RT primers for miR-630, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATT-CAGTTGAGACCTTCCC-3′; specific RT primer for U6, 5′-AAAATATG-GAACGCTTCACGAATTTGC-3′.

2.4. Northern blot

Total RNA (20–30 μg) was resolved on 12.5% denaturing polyacrylamide gel, and electro-transferred to Hybond N+ membranes (Amersham Pharmacia Biotech). After UV cross-link the membranes were prehybridized for 2 h at 42 °C in high efficient hybridized solution (Mylab China), hybridized overnight with Dig-labeled probes of miR-630 (Sangon, Shanghai China), and followed by immunological detection using DIG Detection Kit (Mylab). Blots were visualized by enhanced chemiluminescence regent CDP-star.

2.5. Western blot

Whole-cell extracts were prepared, total proteins (30 μg) in extracts were separated by SDS–PAGE and transferred onto nitrocellulose membranes. The membranes were blocked using TBST buffer containing 5% w/v non-fat milk and incubated with primary antibodies at 4 °C overnight, then immunostained with secondary antibody at RT for 1 h. Primary antibodies were anti-DROSHA (CST-3364, Cell Signaling), anti-E2F1 (CST-3742), anti-β-Actin (PM053, MBL, Nagoya, Japan), and anti-α-Tubulin (PM054) antibodies.

2.6. Luciferase reporter assay

Luciferase activity was measured 48 h after transfection. Luciferase assay was performed using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed according to Upstate ChIP protocol. Antibody was anti-E2F1 antibody (CST3742), immunoglobulin G (IgG) as negative control. The PCR primers used for ChIP assay were 5′-ATGATCCTCCACCACCCA-3′ and 5′-CGTCCTCCAATCCGAAAA-3′.

2.8. Statistical analysis

Student's *t*-test and Wilcoxon's rank-sum test were used for statistical analysis. Statistical significance was defined by a two tailed *p*-value of 0.05.

3. Results

3.1. Enhancement of primary transcript process is involved in miR-630 biosynthesis under CIS exposure

To monitor miR-630 expression under genotoxic stress, human lung cancer A549 cells were exposed to CIS, followed by Northern blot to detect miR-630 and its precursors (Fig. 1A). The levels of pre-miR-630 and mature miR-630 expression in CIS-exposed cells were 13.8- and 7.8-fold of control cells, respectively, while the level of pri-miR-630 was only 2.6-fold of control (Fig. 1B). Alternatively, the ratio between mature miR-630 and pri-miR-630 was 3. The unmatched proportion of pri-miR-630 and mature miR-630 expression was further evidenced by RT-qPCR analysis, in which pri-miR-630 expression under CIS exposure was approximately 2-fold of control ($p = 0.0410$), and the level of mature miR-630

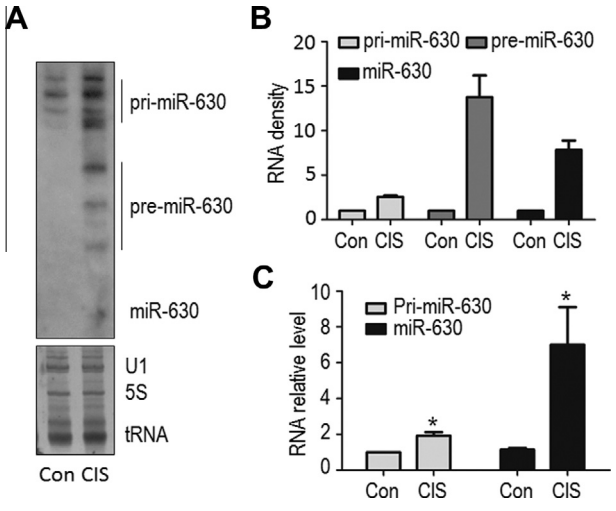

Fig. 1. Cisplatin induces miR-630 and its precursors. A549 cells were exposed to 100 μM CIS for 36 h. Total RNA was isolated. (A) Northern blot analysis of pri-miR-630, pre-miR-630 and miR-630 expression. Following hybridization by Dig-labeled probe corresponding to miR-630, immunological detection was performed using DIG Detection Kit. Ethidium bromide staining of U1, 5SRNA and tRNA serves as loading control. (B) Relative expression of pri-miR-630, pre-mir-630 and miR-630 in (A) experiments. After hybridized with Dig-labeled probe, the Dig-labeled signals were measured by densitometric scan. The level of control RNAs in unexposed cells were normalized to "1". Data present mean ± SD ($n = 3$). (C) RT-qPCR assay of pri-miR-630 and miR-630 expression. The relative level of pri-miR-630 was normalized against GAPDH, and the relative level of miR-630 was normalized against U6. Data present mean ± SD ($n = 3$) $^*p < 0.05$.

was 7-fold of control ($p = 0.0372$) (Fig. 1C), i.e., the mature/pri-miR-630 ratio was 3.5. These data indicate that in addition to transcription activation of the miR-630 gene under CIS exposure, post-transcription processing of pri-miR-630 is most likely to contribute to the unmatched proportion of pri-miR-630 and mature miR-630 expression.

3.2. Induction of DROSHA promotes miR-630 expression in CIS-exposed cancer cells

During miRNA generation, pri-miRNAs are processed by the nuclear DROSHA into pre-miRNAs [5,6], thus the DROSHA protein was presumably induced by CIS exposure. To clarify this idea, DROSHA expression in CIS-exposed cells were examined. As expected, the expression of DROSHA mRNA in CIS-exposed A549 and H1299 cells increased to approximately 2.0–2.5 folds of control cells ($p = 0.0229$ in A549; $p = 0.0443$ in H1299) (Fig. 2A); meanwhile, DROSHA and E2F1 proteins were markedly increased (Fig. 2B). To demonstrate whether the alteration of DROSHA expression upon CIS exposure could influence miR-630 expression, DROSHA expression was silenced by siRNA in A549 (Fig. 2C, upper panel) and H1299 (Fig. 2D, upper panel) and exposed to CIS, followed by testing miR-630. RT-qPCR showed that CIS exposure caused a 4.1-fold and 2.5-fold expression of miR-630 in A549 (Fig. 2C, lower panel) and H1299 (Fig. 2D, lower panel) compared to control cells, respectively (CIS *vs* Con: $p = 0.0014$ in A549; $p = 0.0367$ in H1299); while silencing DROSHA almost completely blocked miR-630 expression in both cells. These data indicate that

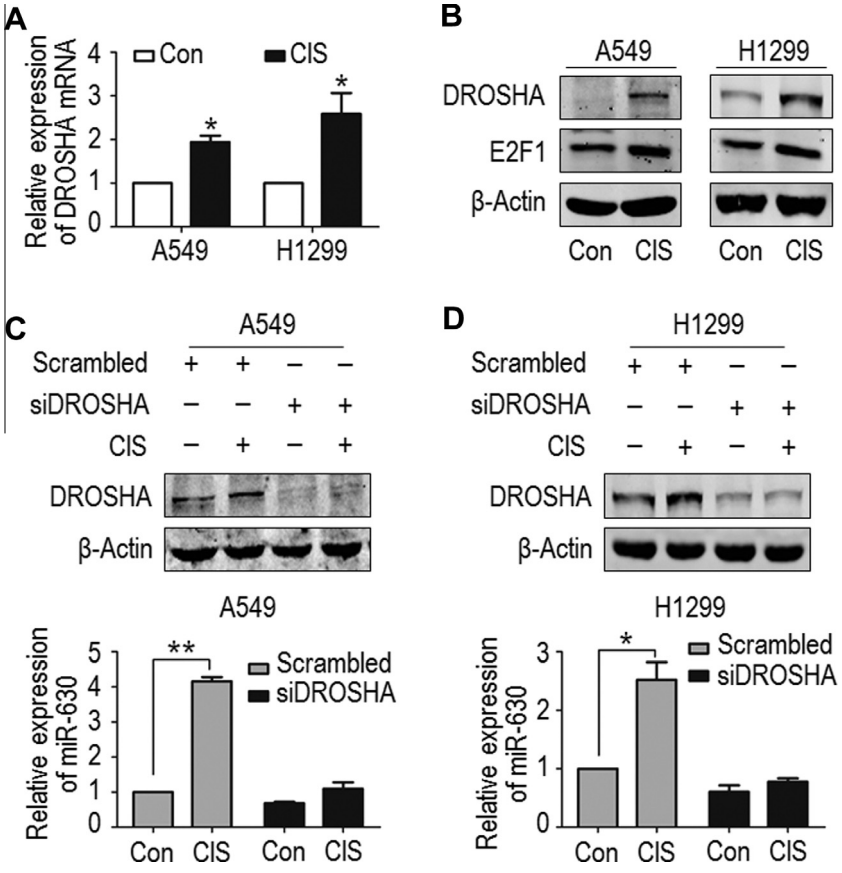

Fig. 2. Induction of DROSHA by CIS promotes miR-630 expression. (A) RT-qPCR analyses for DROSHA mRNA expression. A549 and H1299 cells were exposed to 100 μM CIS for 36 h. GAPDH was used internal control. Data present mean ± SD ($n = 3$). (B) Western blot for DROSHA protein expression in CIS-exposed A549 and H1299 cells in (A) experiments. Specific antibodies were used for blotting DROSHA and E2F1 proteins. β-Actin serves as loading control. (C) and (D) Inhibition of miR-630 expression by silencing DROSHA in CIS-exposed A549 and H1299. Cells were transfected with DROSHA siRNA (scrambled RNA as control). After 48 h transfection cells were exposed to CIS for additional 36 h. Western blot was performed for silencing DROSHA (upper panels in (C and D) and RT-qPCR for miR-630 expression (lower panels). In RT-qPCR for miR-630 expression, U6 is used for normalization. Data present mean ± SD ($n = 3$) $^*p < 0.05$.

induction of DROSHA contributes at least partly to CIS-induced pri-miR-630 and mature miR-630 expression in unmatched proportion. Alternatively, process of pri-miR-630 by DROSHA is involved in the regulation of CIS-induced miR-630 expression.

3.3. E2F1 up-regulates DROSHA expression through direct binding and activating DROSHA promoter under CIS exposure

Since induction of E2F1 accompanied with DROSHA was observed in CIS-exposed A549 and H1299 (Fig. 2B), DROSHA up-regulation might be associated with E2F1 induction under CIS exposure. To answer this question, E2F1 expression plasmid was transfected into A549 for 48 h, followed by detecting DROSHA mRNA and protein expression. RT-qPCR and Western blot showed that both mRNA and protein of DROSHA were up-regulated (Fig. 3A and B), indicating that E2F1 expression may up-regulate DROSHA. To demonstrate E2F1 activation of the *DROSHA* gene under CIS exposure, ChIP assay was performed to examine the *in vivo* binding of E2F1 to the DROSHA promoter. The binding of E2F1 to the region from −292 to −82 of the promoter was markedly increased in CIS-exposed A549 compared to control cells (Fig. 3C), indicating that E2F1 can activate the DROSHA promoter under CIS-induced stress.

To clarify the regulatory mechanism underlying E2F1-dependent *DROSHA* gene activation, a region upstream of the transcriptional initiation site of the *DROSHA* gene extending from −893 to +142 was amplified and sequenced. Bioinformatics analysis showed the DROSHA promoter sequence containing several similarities of transcription regulatory elements including CCAAT box, GC box, and E-box, in particular, two putative E2F binding sites at the positions −214/−207 (TTTGCGCC) and −167/−160 (CCGGGAAA) (Fig. 3D and E). Next, luciferase reporter constructs, in which the *luc* gene expression was driven by 5′-deletion mutants of the DROSHA promoter, were generated (Fig. 3F) and co-transfected with E2F1 expression plasmid into A549 cells. The reporter enzyme assay showed that the −893/+142 promoter reporter construct displayed more than 3.8-fold of control activity (pGL3-basic plasmid). 5′ deletion from −893 to −433, −292, and to −198 reduced approximately 23%, 38% and 62% activities, respectively, compared to the activity of pGL3-DROSHA(−893)-luc (−433 *vs* −893, p = ns; −292 *vs* −893, p = 0.0447; −198 *vs* −893, p = 0.0053), and further, from −198 to −100, the enzyme activity was completely abolished (Fig. 3F). Progressive reduction of activities suggests multiple discrete positively acting elements residing in the promoter; in particular, the sequence from −292 to −101 containing two E2F1 sites at positions −214/−207 and −167/−160 (Fig. 3D and E) is crucial for activating DROSHA promoter. To clarify further the importance of the two E2F1 sites, substitution mutants of the two sites in the sequence from −433 to +142 were generated (Fig. 3G) and inserted into the pGL3-Basic vector to generate pGL3-DROSHA(−433/WT)-Luc and its mutant reporters (MT1, MT2 and MT1/2). Co-transfection with E2F1 expression plasmid and reporter enzyme assay showed that the pGL3-DROSHA(−433/WT)-Luc displayed approximately 2.6-fold of control (pGL3-Basic) activity in E2F1 over-expressed A549. Whereas mutation of −214/−207 site (MT1) decreased approximately 28% activity, mutation of −167/−160 site (MT2) decreased 45%, and mutation of both sites (MT1/2) had no activity, compared to

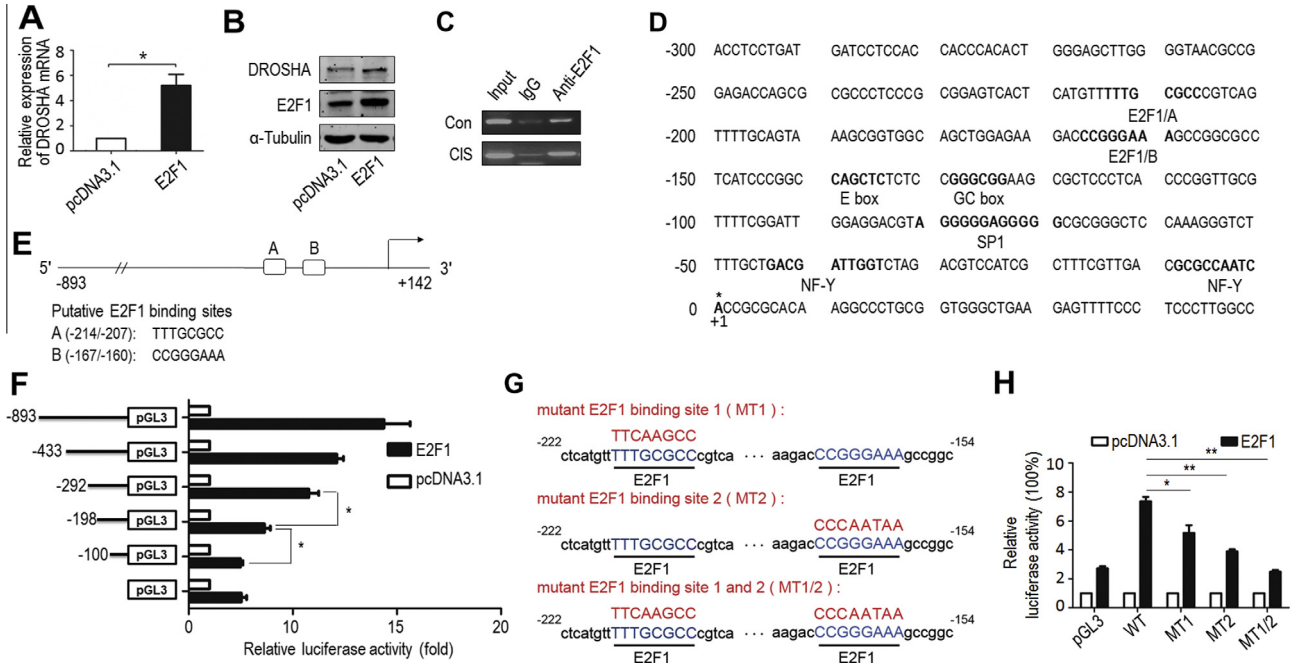

Fig. 3. E2F1 up-regulates DROSHA through binding and activating DROSHA promoter under CIS exposure. (A) The effect of E2F1 expression on DROSHA expression. A549 cells were transfected with pcDNA3.1-E2F1 expression plasmid. After 48 h transfection, total RNAs were isolated and DROSHA mRNA was analyzed by RT-qPCR. GAPDH was used internal control. Data present mean ± SD (n = 3). (B) Western blot analysis of DROSHA protein in (A) experiments. Specific antibodies were used for blotting DROSHA and E2F1 proteins. β-Actin serves as loading control. (C) ChIP assay for E2F1 binding to the DROSHA promoter. A549 cells were exposed to CIS and chromatin was precipitated by anti-E2F1 antibody. IgG serves as negative control. The DROSHA promoter sequence extending from −292 to −82 was amplified by PCR. (D) Sequence surrounding the transcription initiation site of the DROSHA promoter. The start site (+1) is marked by star (*). The putative E2F1 sites, GC-rich, CCAAT, and E box are boldface. (E) Interpretation of the DROSHA promoter. The "A" and "B" indicate the two E2F1 sites. The lower panel shows the two E2F1 site sequences. (F) 5′ Deletion analysis of the DROSHA promoter. The 5′ deletion *Luc*-reporter constructs (200 ng) was transfected into A549, and luciferase activities were assayed 48 h post-transfection. The enzyme activity of pGL3-basic was normalized to "1". Data present mean ± SD (n = 3). (G) The sequences of the two E2F1 sites and mutants used in this study. (H) Substitution mutation analysis of the effects of E2F1 sites on the promoter activation. A549 cells were co-transfected with pGL3-DROSHA(−433/WT)-Luc or mutants (MT1, MT2 and MT1/MT2) with pcDNA3-E2F1 plasmid (200 ng) (pcDNA3.1 empty vector as control). After 48 h transfection, reporter enzyme activities were assayed. The enzyme activity of pGL3-basic was normalized to "1". Data present mean ± SD (n = 3). $^{*}p < 0.05$; $^{**}p < 0.01$.

wild-type construct (Fig. 3H). Taken together, the E2F1 sites at the positions −214/−207 and −167/−160 are crucial for E2F1-dependent *DROSHA* gene activation.

3.4. E2F1 promotes miR-630 expression through DROSHA-mediated processing

To examine the effect of E2F1-induced DROSHA expression on miR-630 expression, pcDNA3.1-E2F1 plasmid was transfected into A549 and H1299 (Fig. 4A), followed by testing mature miR-630 and pri-miR-630 expression. RT-qPCR showed that E2F1 over-expression increased mature miR-630 expression by 3-fold in A549 and one fold in H1299, respectively, compared to control cells (Fig. 4B), whereas over-expression of E2F1 had no any effects on pri-miR-630 levels in both cells (Fig. 4C), indicating that expression of E2F1 promotes mature miR-630 but not pri-miR-630 expression. Alternatively, E2F1 regulates miR-630 gene expression through promoting pri-miR-630 processing. To investigate whether E2F1 promoted miR-630 expression through inducing DROSHA, A549 and H1299 were transfected with DROSHA siRNA to silence DROSHA expression. After 48 h transfection, E2F1 was over-expressed in both cells (Fig. 4D and E, upper panels), and expression of mature miR-630 were detected. Again, the results showed that E2F1 over-expression increased miR-630 levels by 1–2 folds in scrambled RNA-transfected cells (Control *vs* E2F1 transfection: $p = 0.0485$ in A549; $p = 0.0129$ in H1299), but did not have any effects on miR-630 in siRNA-transfected cells (Fig. 4C and D, lower panels), indicating that E2F1 promotes miR-630 biosynthesis through DROSHA-mediated pri-miR-630 processing.

4. Discussion

MiRNA microarray analyses have revealed that some microRNAs are down-regulated and others including miR-630 are up-regulated under CIS exposure [21,22]. It seems likely that activation of microRNA (including miR-630) and *DICER* genes by a certain transcription factor is involved in microRNA synthesis under CIS-induced stress [21]. Here we have demonstrated that E2F1 enhances miR-630 biosynthesis under CIS exposure through promoting DROSHA-mediated pri-miR-630 processing.

The biogenesis of miRNA is a multistep process, and each of these steps is potentially subjected to regulation. DNA damage may regulate miRNA gene expression at transcriptional level, in which transcription factors such as c-Myc [23], p53 [11], and E2F1 [17,18] play roles. DNA damage may also regulate miRNA expression through modulating miRNA processing and maturation [18]. Recently, c-Myc was shown to modulate primary microRNA processing via the transcriptional regulation of DROSHA [24]. Furthermore, association of SMAD proteins with the RNA helicase p68 in the DROSHA microprocessor complex contributes to pri-miRNA processing [25]. It seems likely that the activity of the DROSHA microprocessor complex can be regulated by certain signals. Although transcriptional regulation of miRNA gene by E2F1 upon DNA damage has been revealed [17,18], the role of E2F1 in miRNA processing in response to DNA damage is unknown. We found that E2F1 over-expression could up-regulate DROSHA mRNA and protein expression (Fig. 3A and B), which was accompanied by an increase of mature miR-630 (Fig. 4B) rather than pri-miR-630 (Fig. 4C). Furthermore, silencing DROSHA could completely block the increase of miR-630, although E2F1 was over-expressed

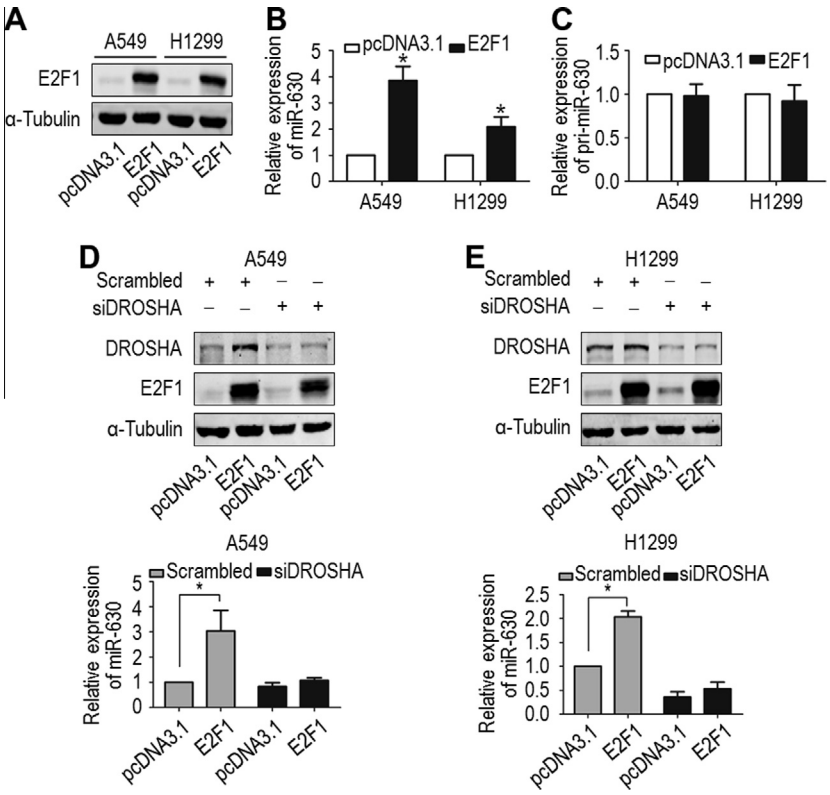

Fig. 4. E2F1 promotes miR-630 synthesis through DROSHA-dependent miRNA processing. (A) Western blot for E2F1 over-expression in A549 and H1299 cells. (B) Enhancement of miR-630 by overexpressed E2F1. A549 and H1299 cells were transfected with E2F1 for 48 h, total RNA was isolated, and RT-qPCR was performed to analyze mature miR-630. (C) RT-qPCR for pri-miR-630 in E2F1-transfected A549 and H1299. pcDNA3.1 empty vector transfection normalized to "1". Data present mean ± SD ($n = 3$). (D) and (E) Inhibition of miR-630 expression by silencing DROSHA in E2F1-overexposed A549 and H1299. Cells were co-transfected with DROSHA siRNA (scrambled RNA as control) and pcDNA3.1-E2F1 plasmid. After 48 h transfection, Western blot was performed for silencing DROSHA and over-expressed E2F1 (upper panels in C and D) and RT-qPCR for miR-630 expression (lower panels). pcDNA3.1 empty vector transfection normalized to "1". Data present mean ± SD ($n = 3$). $^*p < 0.05$.

(Fig. 4D and E). We therefore conclude that E2F1 promotes pri-miR-630 processing through regulating *DROSHA* gene expression. The previous findings [24,25] and our observation support the notion that the activity of DROSHA can be regulated depending on cell conditions. It shows that activation of both miR-630 and *DICER* genes is implicated in phospho-ΔNp63a-regulated miR-630 and other microRNA expression upon CIS exposure [21]. We found that following CIS exposure, the increases of pri-miR-630, pre-miR-630 and mature miR-630 were out of proportion (Fig. 1). Our data indicate that in addition to DROSHA-mediated pri-miR-630 processing, pre-miR-630 processing is also involved in miR-630 synthesis, which is in consistence with the previous finding [21]. Of course, we also cannot exclude the roles of CIS-induced transcription factor in miR-630 gene activation, because the level of pri-miR-630 was elevated in CIS-exposed cells (Fig. 1). As DROSHA is required for pri-miRNA processing [5,6] and E2F1 is frequently induced by DNA damage [15,16,19,20], it seems likely that E2F1-dependent DROSHA activation in primary microRNA processing might be a common event in response to DNA damage.

The DROSHA promoter contains two putative E2F binding sites at the positions −214/−207 and −167/−160 (Fig. 3D and E). Our 5′ deletion mutation and reporter enzyme assays showed that the region from −433 to −101 of the DROSHA promoter was crucial to the promoter activity (Fig. 3F). Furthermore, substitution mutants of the two sites in the sequence from −433 to +142 completely abolished the activity of the promoter (Fig. 3D and E). Our data indicate that the two E2F1 sites are necessary for activating the *DROSHA* gene. In addition, our bioinformatics analysis of the DROSHA promoter revealed neither TATA nor AT-rich elements within this sequence; instead, two SP1 binding sites are located at the positions −29/−24 (GGGCGG) and −81/−70 (AGGGGGAGGGGG), one E box (CANNTG) at the position −81/−76 and two CCAAT boxes (NF-Y binding site) at positions −33/−44 and −9/+7, respectively (Fig. 3D). It is suggested that association of E2F1 with SP1 is a potential mechanism of functional synergy in some promoters [26]. Furthermore, NF-Y recruits RNA Pol II and general transcription factors onto CCAAT box-containing promoters in response to various inductions, thereby permits strong transcriptional activation independently of histone modifications [27]. Therefore, E2F1 interaction with SP1 and its coordination with NF-Y may presumably activate the *DROSHA* gene upon DNA damage.

In summary, we have identified a new role of E2F1 in the regulation of microRNA biosynthesis, in which E2F1-regulated DROSHA expression enhances primary microRNA transcript processing, thereby promoting microRNA synthesis upon DNA damage. The involvement of E2F1-dependent DROSHA activation in pri-miRNA processing under DNA damage stress will provide further insight into the regulation of miRNA biosynthesis. These data also give us a deeper understanding of E2F1role in response to DNA damage.

Acknowledgments

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