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Altered expression of microRNAs in the response to ER stress

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Abstract MicroRNAs, a class of small noncoding RNAs, play key roles in diverse biological and pathological processes. ER stress, resulting from the accumulation of unfolded or misfolded proteins in the ER lumen, is triggered by various physiological events and pathological insults. Here, using RNA deep sequencing analysis, we found that the expression of some microRNAs was altered in HeLa and HEK293 cells under ER stress. Protein and RNA levels of DGCR8, Drosha, Exportin-5, Dicer, and Ago2 showed no significant alteration in ER-stressed cells, which suggested that the change in microRNA expression might not be caused by the microRNA biogenesis pathway but by other, unknown factors. Real-time PCR assays confirmed that hsa-miR-423-5p was up-regulated, whereas hsa-miR-221-3p and hsa-miR-452-5p were down-regulated, in both HeLa and HEK293 cells under ER stress. Luciferase activity and Western blot assays verified that CDKN1A was a direct target of hsa-miR-423-5p and that CDKN1B was a direct target of hsa-miR-221-3p and hsa-miR-452-5p. We speculated that by regulating their targets, microRNAs might function cooperatively as regulators in the adaptive response to ER stress.

Keywords microRNA · Noncoding RNA · ER stress · UPR

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1 Introduction

MicroRNAs (miRNAs) are small (~22 nt), highly conserved noncoding RNAs that bind to target sequences in the 3′ UTR of messenger RNAs (mRNAs) to inhibit translation or promote mRNA degradation, leading to lower protein levels of the target genes. Two key enzymes needed for maturation of these small regulators are the RNase III enzymes, DROSHA, and DICER, which cleave miRNA precursors and eventually lead to the formation of the mature miRNA duplex. To date, more than 1,000 miRNAs have been registered for the human species, and the number is still increasing [1]. It is estimated that one-third of all protein-coding genes are controlled by miRNAs [2]. miRNAs have emerged as key regulators of diverse biological and pathological processes, including cell cycle control, cell growth, differentiation, apoptosis, and embryo development [3]. Accumulating evidence also indicates that miRNA dysfunction leads to human diseases including cancer [4].

The endoplasmic reticulum (ER) is the primary subcellular organelle where proteins are synthesized, modified, and folded. The homeostasis of the ER is indispensable for its normal function. Once it is disturbed by various physiological events or pathological insults, unfolded or misfolded proteins accumulate in the ER lumen, resulting in ER stress. During ER stress, a series of adaptive mechanisms are activated to cope with protein-folding abnormalities, which together are called the unfolded protein response (UPR) [5]. The UPR is mediated by stress sensors such as inositol-requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) [5]. Following ER stress, cells are arrested within G_1 phase [6] and ER stress serves to induce a checkpoint that allows cells to gain time to re-establish homeostasis. The UPR signaling pathway is coordinated

with a decreased rate of protein synthesis and G1 phase arrest. PERK was found to serve as a critical effector of UPR-induced growth arrest, linking stress in the ER to control of cell cycle progression [7]. When cells undergo irreversible ER stress and the homeostasis of the ER cannot be restored, the UPR induces apoptosis of the damaged cells [5].

Recently, an increasing number of studies have verified the function of miRNAs in ER stress [8]. Byrd et al. [9] reported that miR-30c-2* is induced by the PERK pathway of the UPR and governs the expression of XBP1 (X-box binding protein 1), which is a key transcription factor that promotes cell survival in the adaptive UPR, by directly targeting the 3′ UTR of its mRNA. Chitnis et al. [10] demonstrated that PERK induces miR-211 in ER stress, which in turn attenuates stress-dependent expression of the proapoptotic transcription factor CHOP by directly targeting its proximal promoter. They suggested that PERK-dependent miR-211 induction may prevent premature CHOP accumulation and help the cell to re-establish homeostasis prior to apoptotic commitment. miR-322, a miRNA whose expression is down-regulated in ER stress, targets PDIA6, whose increased abundance promotes the sustained activation of IRE1α signaling under ER stress [11]. These observations indicate a key role for miRNAs as critical modulators of the ER stress response.

In this study, we investigated the altered expression of miRNAs in HeLa and HEK293 cells under ER stress and the potential regulators. We speculated that by negatively regulating their targets, these miRNAs might function to mediate the UPR signaling pathway, which is the adaptive response to ER stress. We performed miRNA profiling in HeLa and HEK293 cells under ER stress by small RNA deep sequencing. Through real-time PCR assays, we also validated that the expression of hsa-miR-423-5p and hsa-miR-1246 was up-regulated, whereas that of hsa-miR-221-3p and hsa-miR-452-5p was down-regulated in ER-stressed HeLa and HEK293 cells. CDKN1A plays a role in the shift from the prosurvival to the proapoptotic function of the UPR during ER stress [12], and CDKN1B is a cell cycle inhibitor that prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes [13]. Our luciferase activity and Western blot assays indicated that CDKN1A was a target of hsa-miR-423-5p, and CDKN1B was a direct target of hsa-miR-221-3p and hsa-miR-452-5p. Our data suggested that microRNAs, whose expression was altered in ER stress, might function in the adaptive response to ER stress by regulating their targets.

2 Materials and methods

2.1 Cell culture and ER stress induction

HeLa and HEK293 cells were maintained under standard culture conditions with DMEM plus 10 % FBS at 37 °C and 5 % CO_2. To induce ER stress, cells were treated with 300 nmol/L thapsigargin (TG) and harvested at 6, 12, and 24 h following TG treatment. Ethanol (EtOH), solvent of thapsigargin, was used as control.

2.2 miRNA sequencing

miRNA sequencing was performed with Illumina GAII 2000 with total RNA isolated from cells using Trizol reagent (Invitrogen, Carlsbad, USA). A total of 5,070,156 and 5,169,260 reads were obtained from the control (EtOH-RNA) and sample (TG-RNA) library, respectively, in HeLa cells. In HEK293 cells, 3,611,140 reads were obtained from the control (EtOH-RNA) library and 746,986 reads from the sample (TG-RNA) library. The number of reads for each miRNA was adjusted to reads per million (RPM) to compare the expression levels between the control and sample groups.

2.3 Real-time PCR and primers

Total RNA was isolated from cells using Trizol reagent (Invitrogen) following the manufacturer's instructions, and DNA was eliminated with nuclease-free DNase (Promega, Madison, USA). Complementary DNA (cDNA) was synthesized from RNA (100 ng) of each sample via a reverse transcription reaction with random primers. The cDNA was then used for real-time PCR with SYBR green (TaKaRa Biotechnology, Dalian, China), with GAPDH as internal control. The primers used for reverse transcription and real-time PCR of microRNA are listed in Tables S1 and S2, respectively. The primers used for real-time PCR of mRNA are listed in Table S3.

2.4 Western blot

Whole cell lysate and Western blot analysis were performed as described previously [3]. Protein of cells with or without thapsigargin treatment for 24 h was utilized for Western blotting assay. The primary antibodies used were as follows: mouse polyclonal anti-DGCR8 (1:500) (Sigma, St. Louis, USA), rabbit polyclonal anti-Drosha (1:500) (Sigma), rabbit polyclonal anti-Dicer (1:500) (Sigma), rabbit polyclonal anti-Exportin-5 (1:500) (Sigma), rabbit polyclonal anti-Ago2 (1:500) (Sigma), rabbit polyclonal anti-CDKN1A (1:1,000) (Santa Cruz, Dallas, USA), rabbit polyclonal anti-CDKN1B (1:300) (Sangon, Shanghai, China), and rabbit monoclonal anti-β-actin (1:1,000) (Cell Signaling Technology, Boston, USA). PVDF membranes were incubated for 1 h at room temperature along with primary antibody in 5 % non-fat milk and subsequently with horseradish peroxidase-conjugated secondary antibody. The signal was detected using a chemiluminescence detection kit (PerkinElmer Life Sciences, Boston, MA,

USA), and band density was analyzed with ImageJ software and normalized to β–actin.

2.5 Plasmid construction

For functional analysis of miRNA, partial segments of the mRNA 3′ UTR containing the miRNA binding sequences of CDKN1A (1.5 kb) and CDKN1B (1.3 kb) were PCR-amplified from cDNA made from HEK293 RNA. The forward primer for CDKN1A 3′ UTR amplification is GCTCTAGAAAGCCTGCAGTCCTGGAAGC, and the reverse primer is GCTCTAGAGTGGGAGGAGCTGTGAAAGA. The forward primer for CDKN1B 3′ UTR amplification is GCTCTAGACAGATACATCACTGCTTGATG, and the reverse primer is GCTCTAGATTGGCTCAGTATGCAACCTT. The PCR product was then subcloned into the *XbaI* site downstream of the stop codon in the PGL3-control firefly luciferase reporter vector. miRNA expression plasmids were constructed by inserting DNA fragment containing pre-miRNA coding sequence between the *Hind*III and *Bam*HI sites of pMR-mCherry (Clontech, Mountain View, USA). The primers listed in Table S3 were used for DNA fragment amplification with genomic DNA of HEK293 cells. The correct orientation of 3′ UTR fragments and pre-miRNA coding sequence in the plasmid DNA constructs were further confirmed by sequencing.

2.6 Plasmid transfection and Luciferase activity assay

Plasmid transfection was performed with Lipofectamine 2000 (Invitrogen) according to the supplier's protocol. For luciferase activity assay, cells were transfected in 96-well plates with miRNA expression plasmids or pMR-mCherry control plasmids, pRL-null (Renilla plasmid), and the firefly luciferase constructs carrying the corresponding 3′ UTR. The luciferase activity was measured 24 h post-transfection using the Dual-Luciferase Reporter 1000 System (Promega) based on the manufacturer's protocol. Briefly, cells were lysed with passive lysis buffer at room temperature for 15 min. The luciferase assay buffer II was then added, and firefly luciferase (F-luc) activity was immediately read using a Fluoroskan Ascent FL microplate reader (Thermo Scientific, Waltham, MA, USA). Next, Stop & Glo Buffers with Stop & Glo substrates were added and mixed briefly. Renilla luciferase (R-luc) activity was immediately read. F-luc activity was normalized to R-luc activity to account for variation in transfection efficiency.

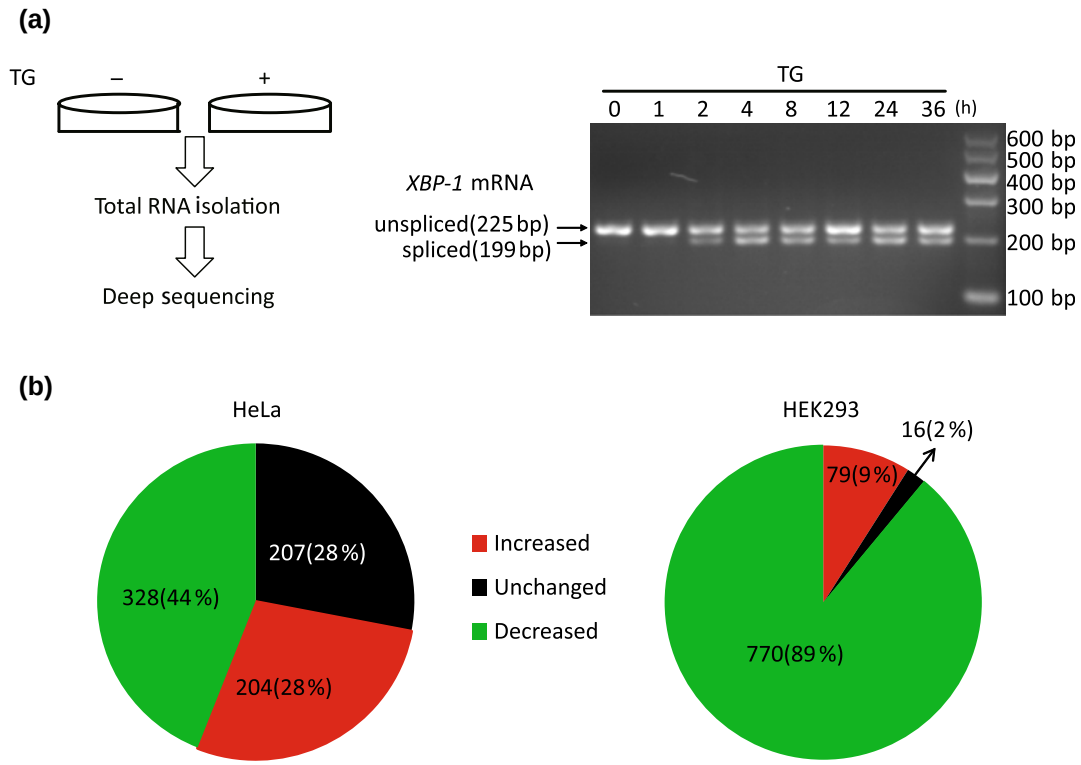

Fig. 1 microRNA expression was altered in ER-stressed cells. **a** (Left) ER stress was induced by 300 nmol/L thapsigargin (TG) in HeLa and HEK293 cells for 24 h; ethanol was used as a control. Total RNA of cells was isolated for high-throughput small RNA deep sequencing. (Right) The cytoplasmic splicing of the *xbp-1* mRNA in response to TG treatment was detected by separating the RT-PCR products in an agarose gel. **b** microRNA expression profile analysis of the deep sequencing results. Number and percentage of increased, unchanged, and decreased microRNAs in ER-stressed HeLa and HEK293 cells

2.7 Statistic analysis

All values reported in this study represent the average of three independent experiments with standard error. After analysis of variance by *f* test, the statistical significance and *P* values were evaluated by Student's *t* test.

3 Results

3.1 miRNA expression profile during ER stress

RNA-seq was applied to detect the expression levels of miRNA in response to ER stress. HeLa and HEK293 cells were treated to induce ER stress with 300 nmol/L of TG, and the induction of the UPR to ER stress was verified by the cytoplasmic splicing of the *xbp-1* mRNA [14]. As expected, 24 h of TG treatment induced ER stress exhibited as increased spliced *xbp-1*. Comparing with the control cells, the expression of ~28 % of miRNAs was increased, ~44 % was down-regulated, and ~28 % was unchanged in ER-stressed HeLa cells. By contrast, the expression of ~9 % of miRNAs was up-regulated, ~2 % was unchanged, and ~89 % was decreased in HEK293 cells in response to ER stress. The top 10 significantly increased or decreased miRNAs in HeLa or HEK293 cells are listed in Table 1. Interestingly, some miRNAs exhibited same pattern of change in response to ER stress. For instance, hsa-miR-4508, has-miR-423-5p, hsa-miR-1291, hsa-miR-616-5p, hsa-miR-3648, hsa-miR-1246, and hsa-miR-129-1-3p increased in both cell lines, whereas hsa-miR-452-5p, hsa-miR-519a-3p, hsa-miR-4687-3p, hsa-miR-4738-3p, hsa-miR-221-3p, hsa-miR-33b-3p, and hsa-miR-4747-5p decreased. Some other miRNAs including hsa-miR-450a-5p, hsa-miR-22-3p, hsa-miR-199b-3p, hsa-miR-720, hsa-miR-1244, hsa-miR-22-5p, and hsa-miR-320b remained unchanged in response to ER stress (data not shown) (Fig. 1a, b).

Using real-time PCR, we further validated the ER stress-induced changes in miRNA expression. Consistent with the deep sequencing data, the expression levels of miR-423-5p and miR-1246 were significantly increased in TG-treated HeLa and HEK293 cells, the levels of miR-199b-3p and miR-320b were not affected, and the levels of miR-221-3p and miR-452-5p were significantly down-regulated in HeLa and HEK293 cells after TG treatment (Fig. 2a, b).

3.2 Expression of miRNA processing factors during ER stress

There are several processing steps from primary transcripts to mature miRNAs. Primary miRNAs are cleaved to produce precursor miRNAs by a nuclear RNase III-type enzyme, Drosha, and its cofactor, DGCR8. Following nuclear processing, precursor miRNAs are exported from the nucleus into the cytoplasm by Exportin-5. These precursors are then cleaved by Dicer and release ~22 bp miRNA duplexes. The resulting RNA duplex is loaded onto RISC, which contains Ago2. One strand (the guide strand) of the RNA duplex remains in RISC and functions as the mature miRNA. To investigate whether the changes in the miRNA expression levels are resulted from the altered expression of key factors in miRNA biogenesis pathway, we analyzed the mRNA expression levels of five miRNA processing factors, including *DGCR8, Drosha, Exportin-5, Dicer,* and *Ago2*, in ER-stressed HEK293 cells after TG treatment for 6, 12, and 24 h. The expression of Drosha was slightly decreased at 6 h, but increased at 12 h after TG treatment (Fig. 3a). *Ago2* mRNA was decreased only after 12-h TG treatment. None of the mRNA levels of any of the five miRNA processing factors were significantly changed (Fig. 3a, right panel) after 24-h TG treatment. Unlike the mRNA expression, Western blots did not detect any change on protein levels of any of the five miRNA processing factors (Fig. 3b). Although ER stress may trigger some fluctuation in the mRNA levels of certain miRNA processing factors, the miRNA biogenesis pathway is generally stable upon ER stress, especially in prolonged

Table 1 Top 10 increased or decreased microRNAs under ER stress

HeLa		HEK293	
miRNA	Fold change ($\log_2^{(TG/EtOH)}$)	miRNA	Fold change ($\log_2^{(TG/EtOH)}$)
Increased			
hsa-miR-4508	2.94	hsa-miR-4508	6.64
hsa-miR-628-3p	2.65	hsa-miR-3687	4.64
hsa-miR-1256	2.00	hsa-miR-4488	4.46
hsa-miR-199b-5p	2.00	hsa-miR-3648	4.29
hsa-miR-33b-5p	2.00	hsa-miR-1248	4.17
hsa-miR-3609	2.00	hsa-miR-1291	3.61
hsa-miR-4645-3p	2.00	hsa-miR-5096	3.58
hsa-miR-1246	1.81	hsa-miR-3651	2.81
hsa-miR-3158-3p	1.80	hsa-miR-1244	2.58
hsa-miR-1291	1.66	hsa-miR-4449	2.58
Decreased			
hsa-miR-4467	−3.32	hsa-miR-125a-3p	−4.86
hsa-miR-548u	−3.17	hsa-miR-3164	−4.52
hsa-miR-500a-5p	−3.00	hsa-miR-4525	−4.49
hsa-miR-500b	−3.00	hsa-miR-3175	−4.46
hsa-miR-675-5p	−2.57	hsa-miR-4742-5p	−4.25
hsa-miR-576-5p	−2.50	hsa-miR-877-5p	−4.10
hsa-miR-4745-3p	−2.32	hsa-miR-556-3p	−4.10
hsa-miR-491-5p	−2.32	hsa-miR-105-3p	−3.91
hsa-miR-130a-5p	−2.32	hsa-miR-100-5p	−3.81
hsa-miR-643	−2.32	hsa-miR-4664-5p	−3.81

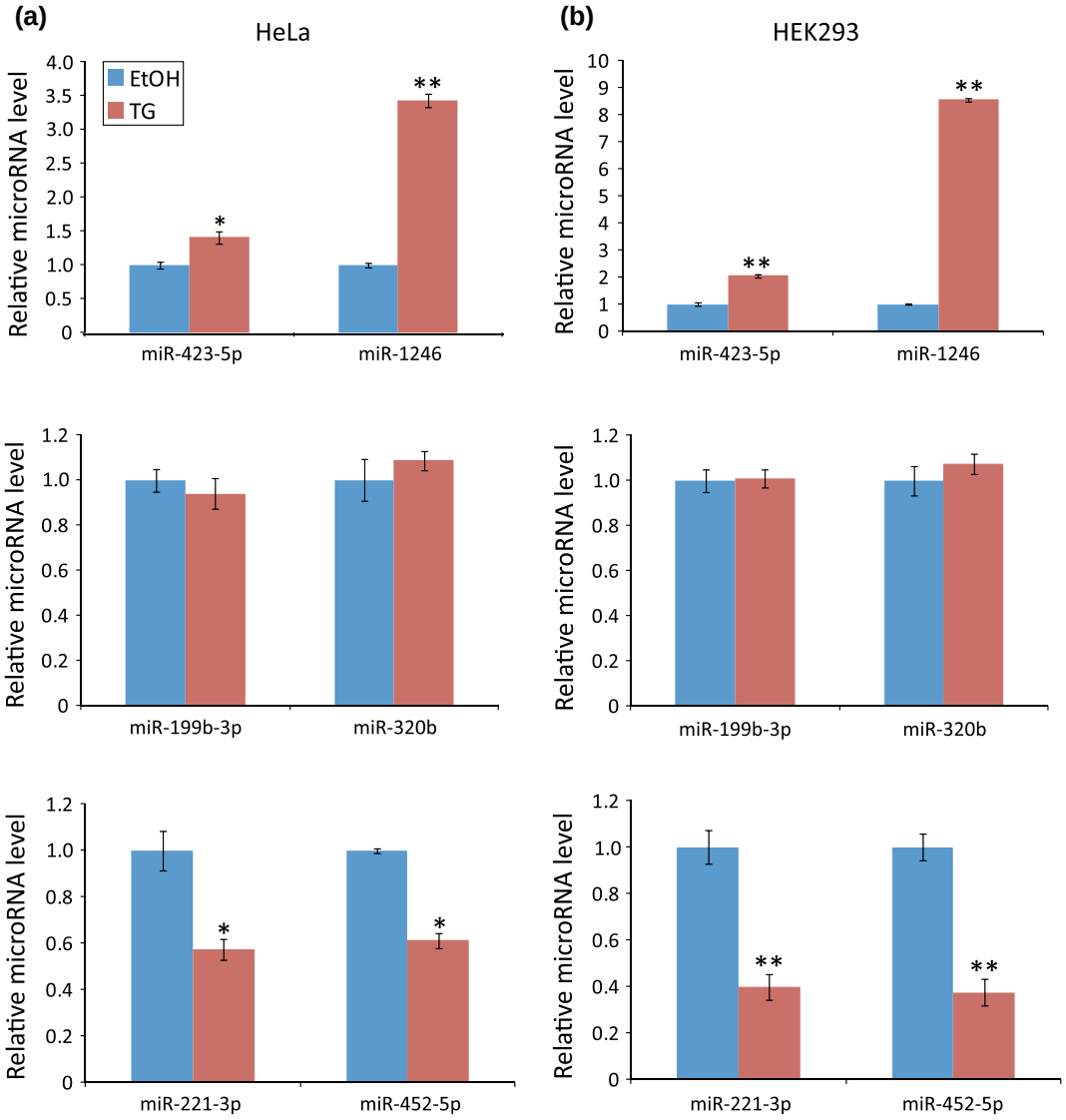

Fig. 2 The microRNA expression profile when under ER stress was validated by RT-real-time PCR. The expression of the increased (miR-423-5p, miR-1246), unchanged (miR-199b-3p, miR320b) and decreased (miR-221-3p, miR-452-5p) microRNAs found in the deep sequencing assay was verified by real-time PCR, as shown in **a** (for HeLa cells) and **b** (for HEK293 cells). Error bars represent the standard error for three independent experiments, and *P* values were determined with a two-tailed Student's *t* test. $*P < 0.05$; $**P < 0.01$

TG treatment (e.g., 24 h) (Fig. 3b). These data indicated that rather than an alteration in the miRNA biogenesis pathway, some other unknown factors might be responsible for the massive changes in miRNA expression levels that were observed in ER-stressed cells.

3.3 Roles of miRNAs in the regulation of *CDKN1B* and *CDKN1A* expression

To further investigate the function of miRNAs in ER stress, we brought our focus on 3 miRNAs, hsa-miR-221-3p, hsa-miR-452-5p, and hsa-miR-423-5p, which showed similar expression profiles in ER-stressed HeLa and HEK293 cells. It is predicted by the *TargetScan* and *miRDB* programs that *CDKN1B* is a potential target of both miR-452-5p and miR-221-3p, and *CDKN1A* is a potential target of miR-423-5p (Fig. 4a). Compared with the control groups, the luciferase activity in HeLa and HEK293 cells transfected with *CDKN1B* 3′ UTR overexpression plasmids was down-regulated by overexpressing miR-221-3p or miR-452-5p (Fig. 4b). Repression of firefly luciferase activity was also observed in HeLa and HEK293 cells co-transfected with reporter plasmids harboring the *CDKN1A* 3′ UTR and plasmids expressing miR-423-5p (Fig. 4b). Furthermore, our Western blot analysis demonstrated that the CDKN1B protein level in the cell was down-regulated by miR-221-

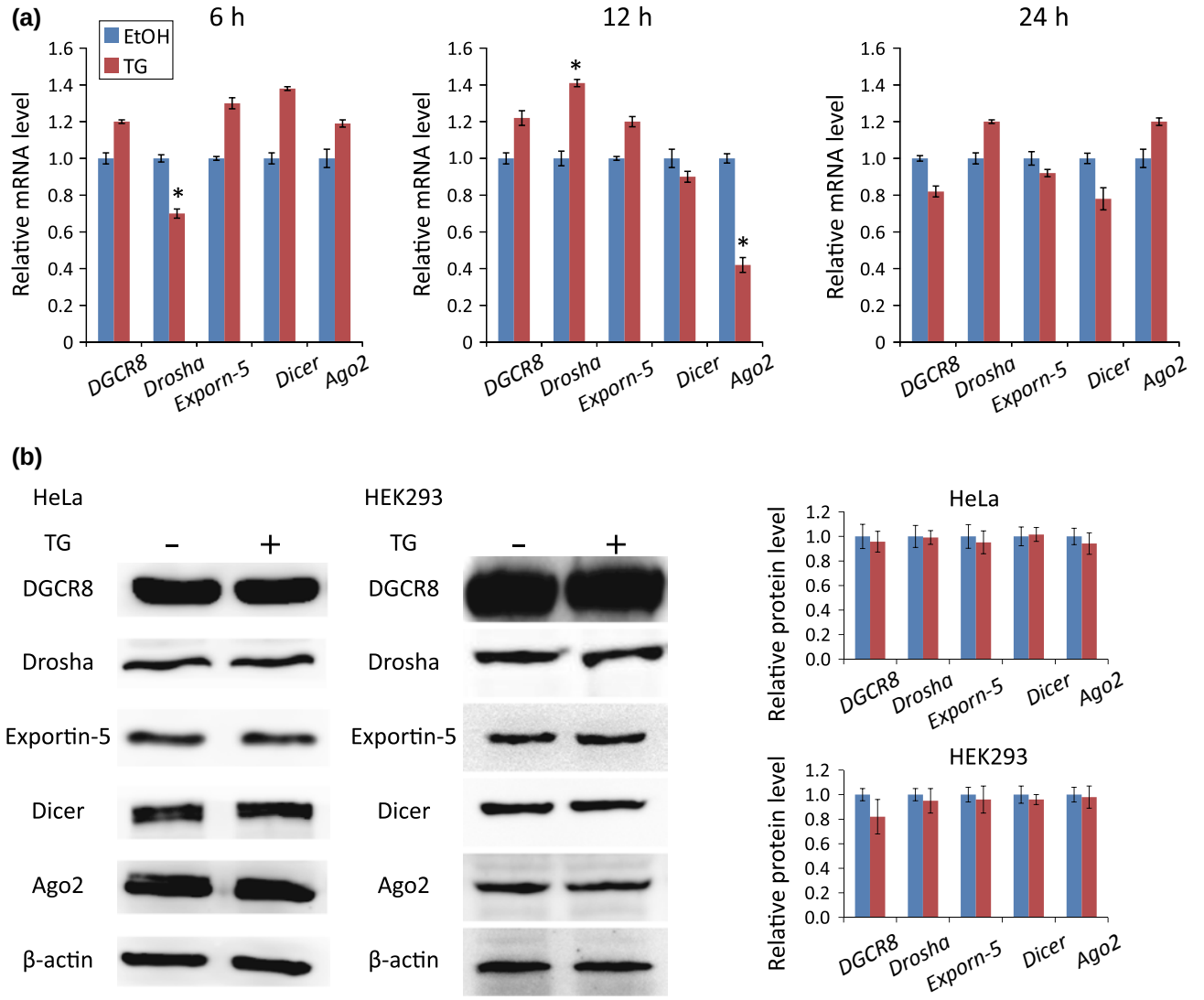

Fig. 3 Examination of the mRNA and protein expression of factors in the microRNA biogenesis pathway. **a** Expression of *DGCR8, Drosha, Exportin-5, Dicer,* and *Ago2* in HEK293 cells under ER stress was detected by real-time PCR. *GAPDH* mRNA was used as a control for normalization. **b** DGCR8, Drosha, Exportin-5, Dicer, and Ago2 protein levels in ER-stressed HeLa and HEK293 cells were tested by Western blot. β-actin protein was used as a loading control, and the relative protein levels were quantified using the ImageJ software (right panel). Error bars represent the standard error for three independent experiments, and *P* values were determined with a two-tailed Student's *t* test. $^*P < 0.05$

3p or miR-452-5p overexpression and that the CDKN1A protein level was decreased by miR-423-5p overexpression (Fig. 4c). These results indicated that *CDKN1A* and *CDKN1B* might serve as direct targets of miR-423-5p, miR-221-3p and miR-452-5p, respectively.

4 Discussion

Accumulation of unfolded or misfolded ER proteins triggers ER stress, and the UPR is primarily a cellular adaptive response that alleviates ER stress by increasing the protein-folding capacity and simultaneously reducing the influx of nascent polypeptides into the ER. miRNAs, which inhibit mRNA translation or promote mRNA degradation by targeting the 3′ UTRs of mRNAs, result in lower protein levels of their target genes. Although increasing evidence has shown co-adjustment between mature miRNAs and signal transducers in the UPR signaling pathway [15–18], little is known about the processing of pri-miRNAs and pre-miRNAs during ER stress.

Our study showed altered expression of a majority of miRNAs in HeLa and HEK293 cells under ER stress (Fig. 1b; Table 1), which suggested the involvement of miRNAs in ER stress. We attempted to determine whether the changes in miRNA expression were attributable to miRNA processing in ER stress. Surprisingly, the expression of several key components of the miRNA biogenesis machinery, including the

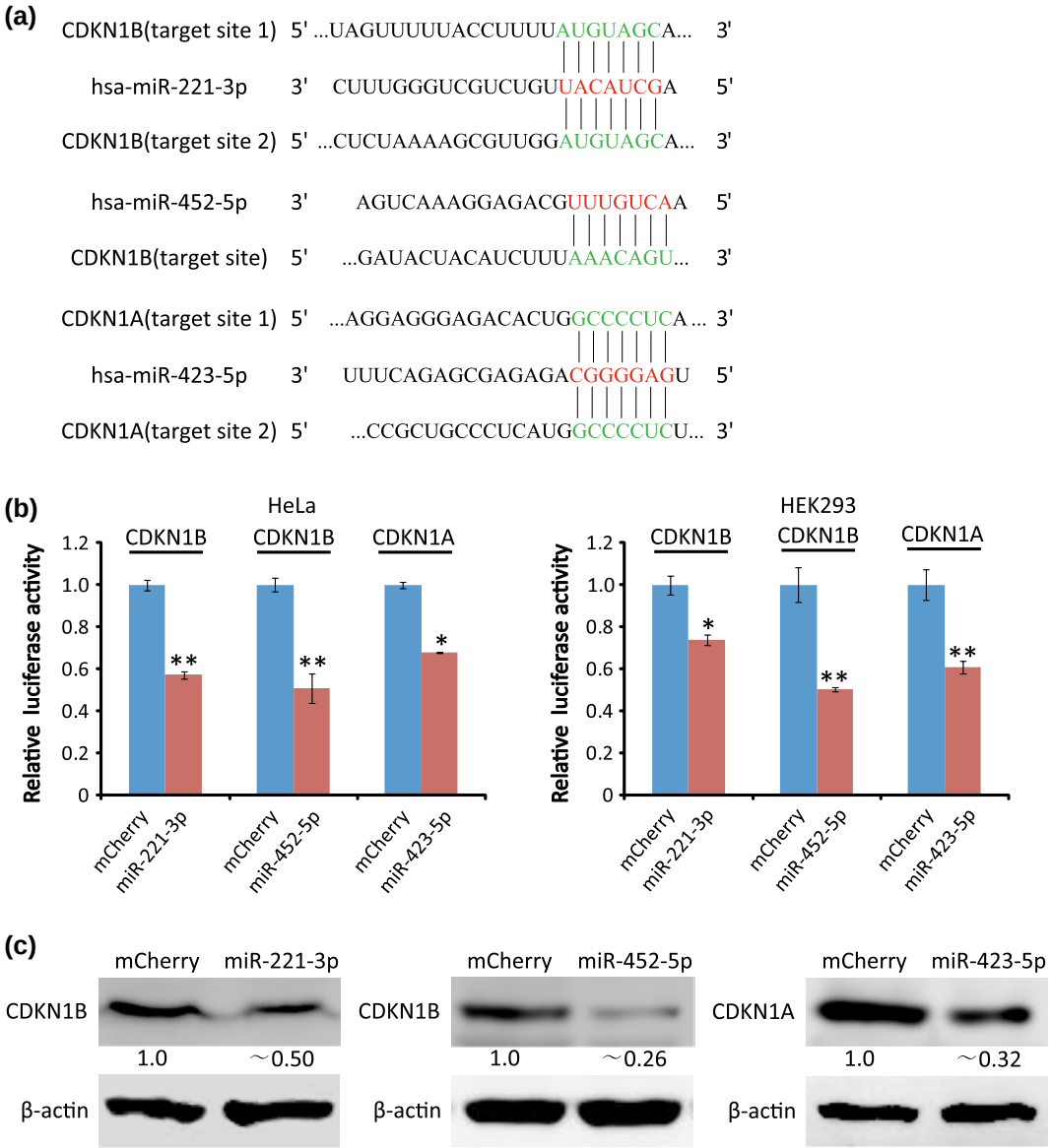

Fig. 4 Validation of microRNA targets by luciferase activity assays and Western blots. **a** There are two potential target sites for hsa-miR-221-3p and one potential target site for hsa-miR-452-5p in the 3′ UTR of the *CDKN1B* mRNA. Two possible target sites for hsa-miR-423-5p are located in the 3′ UTR of the *CDKN1A* mRNA. **b** HeLa and HEK293 cells were co-transfected with microRNA expression plasmids or pMR-mCherry (mCherry) and with the pRL-null (Renilla plasmid) and firefly luciferase reporter plasmids harboring the corresponding 3′ UTR. The ratio of the reporter (*Firefly*) to control plasmids (*Renilla*) in relative luminescence units was plotted. **c** HeLa cells were transfected with the pMR-mCherry control plasmid (mCherry) or microRNA expression plasmids for 48 h and harvested for Western blot analysis of CDKN1B, and CDKN1A. β-actin protein was utilized as a loading control, and the relative protein level was quantified by the ImageJ software. Error bars represent the standard error for three independent experiments, and *P* values were determined with a two-tailed Student's *t* test. $*P < 0.05$; $**P < 0.01$

pri-miRNA processing factors DGCR8 and Drosha, pre-miRNA transferring factor Exportin-5, and pre-miRNA processing factors Dicer and Ago2, fluctuated during ER stress. Moreover, there were no obvious variations in the mRNA or protein levels of any of these genes at 24 h after TG induction, which indicates that miRNA expression changes during ER stress do not result from pri-miRNA or pre-miRNA processing, but may be regulated by unknown factors that affect the stability of mature miRNAs during ER stress. Recently, an increasing number of studies have reported that the stability of a miRNA is regulated by transcription factors or is related to its own sequence. Yu and Hecht [19] found that translin, a DNA/RNA-binding protein, binds to miR-122a and increases its in vivo stability. Bail et al.'s [20] study reported that miR-382, a miRNA that contributes to HIV-1 provirus latency, is unstable in cells and that the 3′ terminus of this miRNA is necessary for its instability. In our future research, we will seek to determine which factors affect miRNA stability in ER

stress, in order to elucidate the mechanism of miRNA expression variation in ER-stressed cells.

How could miRNAs function in ER stress? To address this question, we focused on the study of hsa-miR-221-3p, hsa-miR-452-5p, and hsa-miR-423-5p, whose expression profiles showed high degrees of consistency in ER-stressed HeLa and HEK293 cells. We verified that hsa-miR-423-5p targeted CDKN1A and that hsa-miR-452-5p and hsa-miR-221-3p targeted CDKN1B. Several studies have proposed that CDKN1A may block apoptosis by interacting with proapoptotic molecules such as procaspase-3 in the cytoplasm [12, 21]. CDKN1B, encoding a cyclin-dependent kinase inhibitor, binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes and thus controls cell cycle progression at G1 [22]. By negatively regulating their targets, hsa-miR-423-5p may activate apoptosis, whereas hsa-miR-452-5p and hsa-miR-221-3p may mediate cell cycle arrest. Given the diversity of miRNAs and their targets, our study provides only an example and shows limited information on the regulation of miRNAs during ER stress and the UPR. The down-regulated and up-regulated miRNAs may synergistically function and contribute to the adaptive response to ER stress. The dynamic involvement of miRNAs in ER stress and the UPR is a very complex regulatory network.

Cells are subjected to inner and outer stimuli and insults at all times, and noncoding RNAs are known to play vital roles in these contexts [23–26]. Further investigations are essential to better understand the involvement of noncoding RNAs in these biological events.

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Conflict of interest The authors declare that they have no conflict of interest.

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