microRNA-16 expression in HeLa cells is upregulated in cell-cycle and drug dependent manner

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microRNAs are single-stranded, non-coding RNAs that regulate gene expression. The microRNA-16 family has been reported to be involved in cell-cycle regulation, which could also downregulate expression of multiple pro-proliferation genes. The present results demonstrated that *miR-16* expression in HeLa cells increased when the cells were arrested during S-phase under methyl methanesulfate (MMS) treatment. This further resulted in downregulation of a target protein CDC25A, whereas *miR-16* expression did not increase when HeLa cells were arrested during the MMS-treated G₀/G₁ or G₂/M phase. Furthermore, when HeLa cells were arrested during S-phase with hydroxyurea treatment, *miR-16* expression did not increase. These results suggest that expression levels of microRNAs in mammalian cells are delicately regulated under variable cellular conditions.

microRNA, cell cycle, DNA damage, methyl methanesulfate, HeLa cells

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microRNAs (miRNAs) are single-stranded, non-coding RNAs, ~21 to 23-nucleotides in length, which are generated from larger precursors transcribed from non-coding genes [1]. Results show that miRNAs, as gene regulatory molecules, are involved in many physiological and pathological processes, such as proliferation, differentiation, and tumorigenesis [2]. In addition, the function of miRNAs in mammalian cells has been shown to predominantly decrease target mRNA levels [3]. Furthermore, miRNAs could have a tuning role in regulating gene expression; and the unique features of miRNA-mediated gene regulation have recently been characterized, including the rapid turning off of protein production, as well as reversibly and compartmentalized regulating gene expression [4]. These properties could allow miRNAs to rapidly respond to subtle environmental changes or intracellular stresses.

The miRNA-16 (*miR-16*) family is involved in cell-cycle

regulation, which down-regulates expression of multiple pro-proliferation factors, such as CDK6 and Cyclin D1, resulting in G₀/G₁ arrest [5,6]. Interestingly, cell-cycle inhibition of *miR-16* is cell-type dependent, and over-expression of *miR-16* significantly increases G₀/G₁ population in HCT116, A549, and MCF7 cells, but does not induce a measurable cell-cycle change in HeLa cells [5]. In addition, repression of Cyclin D1 by *miR-16* results in G₀/G₁ arrest of A549 cells, whereas the same effect leads to no G₀/G₁ accumulation in HepG2 or MEG-01 cells [6]. It is possible that *miR-16* targets act in concert, rather than individually, and differential effects are due to various cellular contexts.

The present study analyzed expressional change of *miR-16* levels in HeLa cells at different stages of cell cycle with different DNA-damage reagents. Results showed that *miR-16* expression in HeLa cells was upregulated only when cells were arrested in S-phase under methyl methanesulfate (MMS) treatment.

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1 Materials and methods

1.1 Materials

Hydroxyurea (HU), methyl methanesulfate (MMS), RNase A and propidium iodide (PI) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and DMEM medium were purchased from Invitrogen Life Technologies. HeLa cells were obtained from American Type Culture Collection. CDC25A antibody was purchased from Cell Signaling Technology; β -actin antibodies were purchased from Santa-Cruz; anti-mouse IgG, anti-rabbit IgG and ECL kit were purchased from Amersham.

1.2 Cell culture and cell synchrony

HeLa cells were cultured in Dulbecco's-modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin (Gibco) at 37°C. For synchronization experiments, exponentially growing HeLa cells were initially treated with 2 mmol/L hydroxyurea (HU) for 12 h, were released in a HU-free medium for 10 h, and then subjected to a second round of HU treatment for 12 h. Ultimately, highly synchronized cells were obtained at the G₁/S boundary. For drug-treatment experiments, HeLa cells from the HU double-block were released and subjected to 0.8-mmol/L MMS treatment at the indicated time points.

1.3 Flow cytometric analysis

HeLa cells were harvested and fixed with 70% ethanol at 4°C. The fixed cells were resuspended in a PBS solution containing 500 μ g/mL RNase A and incubated at 37°C for 1 h. Cellular DNA was stained with 30 μ g/mL PI. Flow cytometry analysis was performed with a FACScalibur flow cytometer (BD FACScan).

1.4 Western blot analysis

HeLa cells were lysed in 1× SDS buffer (50 mmol/L Tris, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mmol/L DTT, pH 6.8) and boiled for 10 min. Whole cell lysates were subjected to 10% SDS-PAGE gel electrophoresis, and the proteins were then transferred to PVDF membranes using a semi-dry transfer system (Bio-Rad). After blocking with 10% non-fat milk in TBS-T for 1 h, the membrane was probed with primary monoclonal antibody specific to CDC25A (1:1000) for 90 min, or β -actin (1:2000) for 90 min, which served as an internal control. Membranes were further incubated with anti-mouse IgG or anti-rabbit IgG and detected using an ECL kit.

1.5 RNA extraction and qRT-PCR

Total RNA from cultured HeLa cells was extracted using

Trizol Reagent (Invitrogen) according to manufacture protocols. Reverse transcription was performed using 200 ng total RNA with 0.2 μ L MMLV Reverse Transcriptase (Takara) according to the manufacture protocol. qRT-PCR was performed as described using a real-time PCR Quantitation Kit (GenePharma). PCR reactions were conducted using an ABI3000 real-time PCR machine at 95°C for 3 min, followed by 40 cycles of 95°C for 12 s and 62°C for 50 s. Each sample was analyzed in triplicate, and U6 snRNA was used for normalization. The abundance of *miR-16* in HeLa cells, which was relative to the average expression, was calculated using $RQ=2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T=(C_{T\text{ miRNA}}-C_{T\text{ U6 RNA}})_{\text{experimental}}-(C_{T\text{ miRNA}}-C_{T\text{ U6 RNA}})_{\text{control}}$.

1.6 Anti-*miR-16* transfection

Equal numbers of HeLa cells (3×10^4) were seeded into 6-well culture plates and cultured for 1 d, followed by incubation with 2 mmol/L HU for 12 h. The HU-treated cells were then incubated with fresh medium and transiently transfected with synthesized anti-*miR-16* oligonucleotides (*miR-16* inhibitor) at a final concentration of 100, 200 and 400 nmol/L using Lipofectamine 2000 reagent (Invitrogen), respectively, for 6 h. Synthesized random oligonucleotides served as the *miR-16* negative control. Following transfection, the transfectants were re-cultured with fresh medium for an additional 4 h, treated with 2 mmol/L HU for 12 h, and subjected to further analysis.

2 Results and discussion

2.1 S-phase dependent *miR-16* upregulation in MMS-treated HeLa cells

We recently reported that cell-cycle progression of HeLa cells could be arrested with a DNA damaging alkylating agent MMS, showing that cells at different phases exhibit different sensitivity to MMS treatment [7]. The same observation was shown in Figure 1(c): S-phase HeLa cells (3-h release from HU-double block; see Materials and methods) were fully arrested with 0.8 mmol/L MMS treatment, G₂/M-phase cells (7-h release) were partially delayed, and G₁-phase cells (12-h release) were arrested at G₀/G₁ phase under the same MMS treatment. Interestingly, data from real-time PCR analysis revealed that MMS treatment significantly increased *miR-16* expression in S-phase-arrested HeLa cells, whereas *miR-16* upregulation was not observed in G₂/M-phase and G₀/G₁-phase arrested HeLa cells under the same MMS treatment (Figure 1(d)). *miR-16* expression in different phases of untreated HeLa cells (Figure 1(a)) was also measured and served as the control, of which expression levels at different phases did not reveal significant variation (Figure 1(b)). In summary, these results suggest that *miR-16* upregulation in MMS-treated HeLa cells is S-phase-dependent.

Figure 1 S-phase dependent upregulation of *miR-16* expression in MMS-treated HeLa cells. (a) HeLa cells were synchronized at the G_1/S boundary using the HU double-block method. Synchronized HeLa cells were released from the HU-block for 3 h and represented S-phase cells, for 7 h and represented G_2/M -phase cells, and for 12 h and represented G_1 -phase cells. Cellular DNA contents were analyzed by flow cytometry. Arrowheads indicate 2N and 4N DNA contents. (b) *miR-16* expression in exponentially growing HeLa cells and HeLa cells at different phases, as detected by qRT-PCR. U6 snRNA was used to normalize. Data are representative of 3 independent experiments (mean $\pm$ SD). (c) Synchronized HeLa cells were released from HU-block for the indicated times. Subsequently, the cells were treated with 0.8 mmol/L MMS: the 3-h-released cells (S-phase cells) were treated with MMS for 4 h, the 7-h-released cells (G_2/M -phase cells) were treated with MMS for 5 h, and the 12-h-released cells (G_1 -phase cells) were treated with MMS for 6 h. Cellular DNA contents were analyzed by flow cytometry. Arrowheads indicate 2N and 4N DNA contents. (d) *miR-16* expression in HeLa cells at different phases, with or without MMS treatment, was detected by qRT-PCR. U6 snRNA was used to normalize. Data are representative of 3 independent experiments (mean $\pm$ SD). Asterisk indicates $P < 0.05$.

2.2 *miR-16* upregulation results in decreased CDC25A expression

Because CDC25A, an cell-cycle regulator in S phase [8], was reported to be negatively regulated by *miR-16* [5,9], expression levels of CDC25A protein in S-phase HeLa cells were measured with or without MMS treatment by Western blot analysis. Results revealed significantly downregulated CDC25A protein in S-phase-arrested HeLa cells treated with MMS (Figure 2(a) and (b)), suggesting that upregulation of *miR-16* expression in S-phase-arrested cells resulted in down-regulated CDC25A expression. This was in agreement with a

Figure 2 Up-regulation of *miR-16* causes functionally decreased CDC25A proteins. (a) Western blot analysis of CDC25A in HeLa cells (lane 1, exponentially growing cells (control); lane 2, S-phase cells; lane 3, S-phase cells treated with 0.8 mmol/L MMS for 4 h). β -actin was used as the loading control. (b) ImageJ software was used to analyze relative CDC25A levels from the western blot results in (a). Data are representative of 3 independent experiments (mean $\pm$ SD). Asterisk indicates $P < 0.05$.

previous report that downregulated CDC25A transcription levels were solely dependent on UV-induced *miR-16* in a *miR-16* knockdown experiment (see Figure 5D of ref. [9]).

The regulatory relationship between *miR-16* and CDC25A was further confirmed using synthesized anti-*miR-16* oligonucleotides (Materials and methods). Results showed that inhibition of *miR-16* upregulation by the *miR-16* inhibitor rescued the downregulation of CDC25A expression in the MMS-induced S-phase-arrested HeLa cells (Figure 3(a) and (b)). These results suggest that *miR-16* directly regulate CDC25A expression, which is supported by a previous luciferase activity experiment with a RLuc-CDC25A-3'UTR fragment [9]. However, recovered CDC25A expression in the presence of the *miR-16* inhibitor did not release the MMS-induced inhibitory effects of S-phase progression (data not shown), implying that rescue of CDC25A downregulation by *miR-16* alone might not be sufficient for recovery of MMS-induced S-phase-arrested HeLa cells. These results indicate that specific upregulation of *miR-16* expression in MMS-treated S-phase-arrested HeLa cells could functionally downregulate targets such as CDC25A.

2.3 *miR-16* expression is not upregulated in HU-induced S-phase-arrested HeLa cells

Hydroxyurea (HU) is an inhibitor of ribonucleotide reductase

Figure 3 Inhibition of *miR-16* upregulation rescues downregulation of CDC25A. (a) HeLa cells were transiently transfected with *miR-16* inhibitor (100, 200 and 400 nmol/L) or *miR-16* negative control, as described in Material and methods. *miR-16* expression in these cells was detected by qRT-PCR. U6 snRNA was used to normalize. Asterisk indicates $P < 0.05$. (b) Western blot analysis of CDC25A in HeLa cells (lane 1, exponentially growing cells; lane 2, S-phase cells; lane 3, S-phase cells treated with MMS for 4 h; lane 4, S-phase cells transfected with the *miR-16* negative control (200 nmol/L) and treated with MMS for 4 h; lanes 5–7, S-phase cells transfected with the *miR-16* inhibitor (100, 200 and 400 nmol/L, respectively) and treated with MMS for 4 h. β -actin was used as the loading control.

and has been widely used for synchronizing cells at the G₁/S point, but it also induces DNA strand breaks in mammalian cells [10]. Our previous work showed that longer exposure to HU resulted in higher levels of DNA damage in CHO cells, as well as S-phase arrest [11]. In the present study, S-phase HeLa cells were treated with indicated concentrations of HU for 4 h, respectively. The HU treatments resulted in arrest of HeLa cells at the S-phase (Figure 4(a)). However, real-time PCR analysis did not detect significant upregulation of *miR-16* expression in HU-induced S-phase-arrested cells (Figure 4(b)). Furthermore, Western blot analysis revealed similar protein levels of CDC25A between untreated S-phase HeLa cells and HU-treated S-phase cells (Figure 4(c)). In conclusion, these results showed that MMS treatment resulted in upregulation of *miR-16* expression, but HU treatment did not induce this effect, although both drug-treatments resulted in S-phase arrest of HeLa cells.

It is widely recognized that different kinds of DNA damage during S-phase result in differential activation of various signaling pathways in the DNA damage checkpoint [12–14]. As a widely-used DNA damaging alkylating agent, MMS adds methyl groups to DNA at 7-guanine or 3-adenine, thereby inducing structural damage or DNA single-stranded breaks [15], which often activate the base excision repair pathway [16]. In contrast, HU treatment causes DNA double-stranded breaks in mammalian cells, as well replication disturbances [10–12], which induces activation

Figure 4 *miR-16* expression in S-phase HeLa cells treated with HU. (a) S-phase cells were treated respectively with 2, 4 and 8 mmol/L HU for 4 h, and subjected to flow cytometry analysis. Arrowheads indicate 2N and 4N DNA contents. (b) S-phase cells were treated with 2, 4 and 8 mmol/L HU for 4 h, respectively, and then *miR-16* expression was detected by qRT-PCR. U6 snRNA was used to normalize. Data are representative of 3 independent experiments (mean $\pm$ SD). (c) Western blot analysis of CDC25A of HeLa cells (lane 1, exponentially growing cells; lane 2, S-phase cells; lanes 3–5, S-phase cells treated with HU (2, 4, and 8 mmol/L, respectively) for 4 h; β -actin served as the loading control.

of the ATM/Chk2 pathway [17,18]. It has been reported that MMS activates the p21-dependent S-phase DNA damage checkpoint, but suppresses the CDC25A-dependent checkpoint, whereas UV irradiation results in entirely opposite cellular responses within the same cells [19]. Based on these observations, it is possible that upregulation of *miR-16* expression relies on a particular cellular context determined by MMS-induced responses in terms of the S-phase checkpoint selection, whereas no such cellular context is generated under by HU treatment.

In conclusion, the present study demonstrates that expression levels of miRNAs in mammalian cells are delicately regulated under variable cellular contexts, which are determined by particular stages of the cell cycle and/or by different environmental stresses.

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