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Analysis of microRNA expression profiles during the cell cycle in synchronized HeLa cells

Jue-Yu Zhou[1], Wen-Li Ma[1,], Shuang Liang[1], Ye Zeng[2], Rong Shi[1], Hai-Lang Yu[1], Wei-Wei Xiao[1] & Wen-Ling Zheng[3]*

[1]Institute of Genetic Engineering, [2]Department of Stomatology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, [3]Southern China Genomics Research Center, Guangzhou 510800, P. R. China

Cell cycle progression is regulated by both transcriptional and post-transcriptional mechanisms. MicroRNAs (miRNAs) emerge as a new class of small non-coding RNA regulators of cell cycle as recent evidence suggests. It is hypothesized that expression of specific miRNAs oscillates orderly along with cell cycle progression. However, the oscillated expression patterns of many candidate miRNAs have yet to be determined. Here, we describe miRNA expression profiling in double-thymidine synchronized HeLa cells as cell cycle progresses. Twenty-five differentially expressed miRNAs were classified into five groups based on their cell cycle-dependent expression patterns. The cyclic expression of six miRNAs (miR-221, let-7a, miR-21, miR-34a, miR-24, miR-376b) was validated by real-time quantitative RT-PCR (qRT-PCR). These results suggest that specific miRNAs, along with other key factors are required for maintaining and regulating proper cell cycle progression. The study deepens our understanding on cell cycle regulation. [BMB reports 2009; 42(9): 593-598]

INTRODUCTION

Proper regulation of cell cycle is likely to govern critical aspects of diverse processes, while dysregulation of cell cycle is likely to lead to proliferative diseases, most notably cancer (1). Indeed, previous studies suggested that a large number of genes would be regulated in a cell-cycle-specific manner in normal and cancer cells (2-4). Thus, a global perspective on gene regulation throughout the cell cycle would expand our understanding of both normal cell division and the abnormal phenotypes observed in certain pathological processes.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that are believed to be important in many biological processes by regulating gene expression via degradation or translational inhibition of target mRNAs. Numerous miRNAs and their targets have been identified in many species. Although the functional aspects of most miRNAs remain unknown or obscure, a small number of miRNAs have been shown to play important roles in cell cycle progression (5-8). If miRNAs have control over cell cycle, it is anticipated that their cyclic expression should be readily observed in a timely manner (9). Investigating miRNAs expression pattern in different cell cycle phases would provide insights into miRNA-dependent regulation of cell cycle progression.

In the present study, we sought to investigate the expression patterns of miRNAs throughout the cell cycle in synchronizing HeLa cells treated by double-thymidine block. Twenty-five miRNAs were detected to be differentially expressed and classified into five groups based on their distinct cyclic expression patterns. Our findings support the notion that specific miRNAs, along with other factors, function in cell cycle to achieve proper regulation, which might represent a novel mechanism for regulating cell cycle progression.

RESULTS AND DISCUSSION

The study of cell cycle-specific regulation requires cell cultures growing in the same phase of the cycle. The most commonly used cell cycle synchronization procedures include selection methods such as elutriation and mitotic shake off, and induction methods such as double thymidine block, serum starvation and drug-induced cell cycle arrest (10). However, the sensitivity with which one can detect periodic activities in synchronized cell cultures depends almost entirely on the degree to which cells can be synchronized (4). To identify miRNA activity throughout the cell cycle, here we used HeLa cells, a epithelial cell line derived from cervical carcinoma, which has been widely used for cell cycle studies due to its robust cell cycle synchronization. A double thymidine block was utilized to arrest cells at the G1/S boundary, then the complete cell population traversed the cell cycle at a similar rate following release from thymidine arrest. Synchronized population of cells was obtained at 12 time points every 2 h after the release, and cell cycle was monitored by flow cytometry (FACS) analysis.

Fig. 1 illustrated the cell cycle distribution based on the DNA content in HeLa cells growing asynchronously or

*Corresponding author. Tel: 86-20-62789098; Fax: 86-20-62789097; E-mail: zhoujueyu@126.com, wenli668@gmail.com

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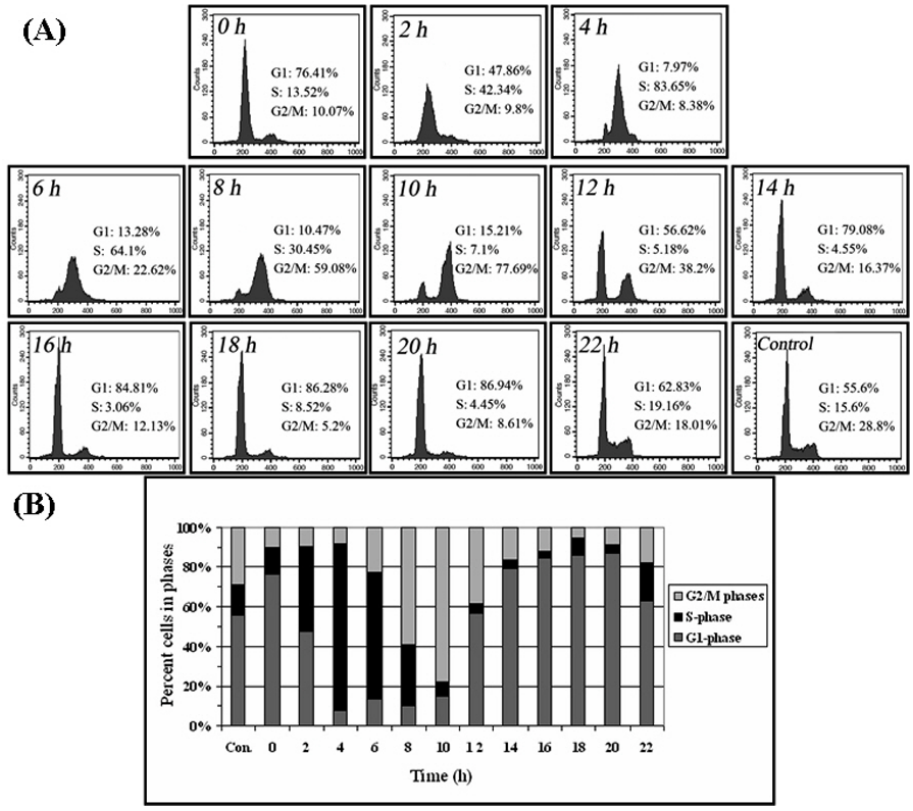

Fig. 1. The synchronization of HeLa cells by double-thymidine block. The percentages of the cells in each phases of the cell cycle were determined by flow cytometry analysis. The results are those of a typical experiment. (A) DNA content frequency histograms representing exponentially and asynchronously growing cells in the control culture (Con.) and synchronized cell populations at different time points. (B) The percentage of cells at different phases of the cell cycle after release from thymidine treatment.

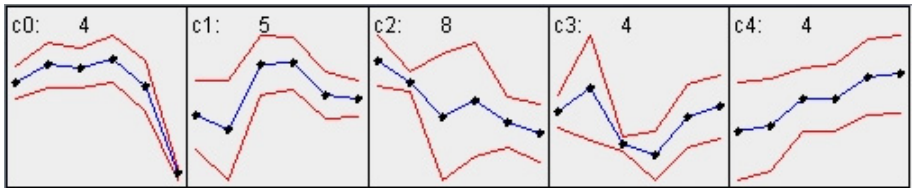

Fig. 2. Five groups based on cell cycle-dependent expression patterns of differentially expressed miRNAs.

synchronized by the double-thymidine block. The percentage of cell cycle phase of asynchronously growing cells (general HeLa cells) was 55.6% in G1 phase, 28.8% in G2/M phase, 15.6% in S phase (Fig. 1A, Control). As evident from the DNA content frequency histograms (Fig. 1B) and actual percentage of cells in respective phases of the cell cycle (Fig. 1A), the cells were effectively synchronized upon entrance to S phase following the double-thymidine block. At the time of release from the block, nearly all cells were residing in the G1/S boundary. Upon release from the thymidine block, the synchrony was maintained throughout the cell cycle. Thus, most of the cells (~80%) were progressing through S phase 4 h after removal of thymidine from the medium, and most cells were entering G2/M phase at about 6 to 10 h and re-entering G1 phase between 12 and 16 h after release.

To compare the expression levels of miRNAs at different phases of the cell cycle in synchronized HeLa cells, total RNA was isolated from HeLa cells growing asynchronously (control) and synchronized at G1 (16 h), S (4 h) or G2/M (10 h) phase, respectively. The quality and yield of the total RNA were satisfactory when assessed with Agilent 2100 Bioanalyzer.

Recent studies documented specific miRNAs in cell cycle regulation and suggested that dysfunction of miRNA-mediated cell-cycle control contributes to malignancies (11). To address this question, the genome-wide expression profiling of miRNAs in synchronized HeLa cells was investigated using a microarray containing 698 mature human miRNA oligonucleotide probes. Twenty-five differentially expressed miRNAs showing cell-cycle-dependent patterns were identified and classified into five groups based on their expression patterns (Fig. 2). These five groups and the fold changes of each differentially expressed miRNA are listed in Table 1. Strikingly, some miRNAs in group C3 and C4 has been reported to participate in cell cycle control and tumorigenesis.

Table 1. Classification of differentially expressed miRNAs during the cell cycle in HeLa cells

miRNA Name	Group	Microarray		
		S/G1	G2M/G1	G2M/S
hsa-miR-19a	C0	1.28	0.11	0.09
hsa-miR-34b	C0	1.48	0.15	0.10
hsa-miR-34c-3p	C0	1.35	0.19	0.14
hsa-miR-933	C0	1.62	0.11	0.07
hsa-miR-329	C1	3.05	1.76	0.58
hsa-miR-519d	C1	2.29	1.30	0.57
hsa-miR-590-3p	C1	2.58	1.22	0.47
hsa-miR-618	C1	1.80	0.79	0.44
hsa-miR-924	C1	3.17	3.52	1.11
hsa-miR-126*	C2	0.36	0.55	1.53
hsa-miR-147b	C2	1.18	0.37	0.31
hsa-miR-224	C2	0.29	0.82	2.77
hsa-miR-299-5p	C2	0.98	0.45	0.46
hsa-miR-524-5p	C2	1.69	0.70	0.42
hsa-miR-553	C2	1.05	0.47	0.45
hsa-miR-582-5p	C2	0.43	0.60	1.37
hsa-miR-653	C2	0.37	0.39	1.06
hsa-miR-34a	C3	0.64	3.71	5.80
hsa-miR-30b	C3	0.50	0.43	0.87
hsa-miR-22	C3	0.12	0.22	1.82
hsa-miR-510	C3	0.26	0.94	3.60
hsa-let-7a	C4	0.79	2.33	2.94
hsa-miR-21	C4	2.63	3.95	1.50
hsa-miR-221	C4	3.82	5.23	1.37
hsa-miR-222	C4	2.14	3.44	1.60

Expression profiling revealed that the expression of miR-221, miR-222 and miR-21 was low at G1, started to increase at S phase, and peaked as cells enter G2/M. Interestingly, miR-221 and miR-222 have been shown to be oncogenic and up-regulated in several tumor-derived cell lines, as well as in cancer patients (12, 13). Furthermore, miR-221 and miR-222 are up-regulated upon exit from quiescence and they are growth regulatory mediators that coordinately modulate the levels of two critical inhibitors (p27 and p57) of CDK2/cyclin complexes in late G1 when competency for cell cycle progression is monitored (6). Moreover, by controlling these two CDK inhibitors, up-regulation of miR-221 and miR-222 can promote the growth of human hepatocellular carcinoma cells and thyroid papillary carcinoma cell line (TPC-1) by increasing the number of cells in S-phase (14, 15). Similarly, miR-21 also functions as an oncogene affecting tumorigenesis, in part through regulation of the tumor suppressor genes TPM1 (16), PDCD4 (17), PTEN (18) and the anti-apoptotic protein BCL2 (19). Additionally, down-regulation of miR-21 in glioblastoma cells has been shown to suppress cancer cell growth, increased apoptosis, and cell cycle arrest (20). Together with our findings that miR-221, miR-222 and miR-21 are significantly up-regulated as cells progress through the G1/S transition, these results indicate that up-regulation of either miR might promote G1/S transition and cell proliferation, whereas down-regulation of these miRNAs might cause cell cycle arrest and inhibit cancer cell growth.

In contrast to miR-221, miR-222 and miR-21, expression of let-7a and miR-34a was down-regulated during G1 and S phases with minimal levels of expression in S phase, especially for let-7a. However, they both were then significantly up-regulated when cells entered G2/M. It is notable that the let-7 miRNA is a founding member of the miRNA family, which controls the timing of cell cycle exit and terminal differentiation in *Caenorhabditis elegans* (21). Furthermore, previous studies have also shown that let-7 miRNA family functions as tumor suppressors by inhibiting the mRNAs of well characterized oncogenes, such as the Ras family (22), HMGA2 (23), c-Myc (24, 25), IMP-1 (26), and cell cycle regulators including CDC25A, CDK6, and CCND2 (27). Introduction of let-7a into liver cancer cells causes growth inhibition and cell cycle changes (27). Likewise, miR-34a, located at 1p36 in the human genome, is frequently deleted in diverse malignancies, pointing to this miRNA as a bona fide tumor suppressor (11). Several groups have demonstrated that miR-34a as a miRNA component of the p53 network and as a direct transcriptional targets of p53 (19, 28, 29). Further evidence has revealed that miR-34a induces G1 arrest by regulation of several cell cycle genes, including CCNE2, CDK4 and hepatocyte growth factor receptor (MET) (19). Sun *et al* suggested that miR-34a triggers G1 arrest in A549 cells by regulating other downstream effectors including CCND1 and CDK6 (5). In addition, ectopic expression of miR-34a reduces the levels of E2F3 by targeting its mRNA (30). The anti-apoptotic protein BCL2 is down-regulated by miR-34a in several cell types, which is consistent with a role for miR-34a in p53-mediated apoptosis (19). In summary, numerous lines of evidence implicate let-7a or miR-34a as major regulators of the cell cycle and of the G1-to-S transition in particular, and their inactivation might contribute to the development of certain cancers. Significantly, these functional aspects of let-7a or miR-34a on cell cycle and oncogenesis are consistent with our findings.

To validate the microarray results, qRT-PCR was performed on six selected miRNAs including four differentially expressed miRNAs (hsa-let-7a, hsa-miR-21, hsa-miR-221, hsa-miR-34a) and two miRNAs (hsa-miR-24, hsa-miR-376b) whose expression remain largely unchanged. The expression levels of these miRNAs were calculated relative to the expression at G1 (Fig. 3). In general, the results of qRT-PCR were all in accordance with the microarray data.

In conclusions, the focus of this study was to identify specific miRNAs regulating the cell cycle in HeLa cells. The results demonstrated for the first time that at least 25 miRNAs expressed in a cell cycle-dependent manner. Our data supports the involvement of cell cycle-regulating miRNAs and demands for further investigation on how these miRNAs might

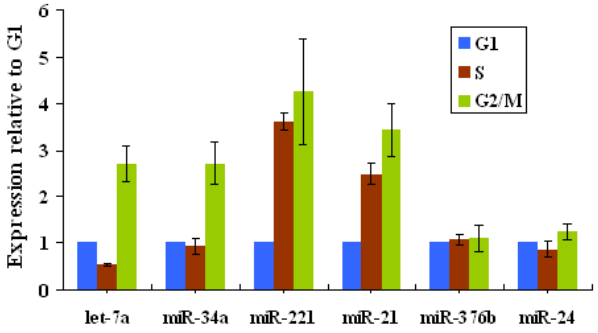

Fig. 3. Relative expression levels of let-7a, miR-34a, miR-221, miR-21, miR-376b, miR-24. Relative expression levels were calculated according to the $2^{-\Delta\Delta C_T}$ method, using 5S rRNA as an internal reference gene and G1 phase as calibrator (relative expression = 1). Data represent the average of three independent experiments ± 1SD.

participate in cell cycle process and tumorigenesis.

MATERIALS AND METHODS

Cell line and cell culture conditions

The human cervical carcinoma cell line HeLa was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cell line was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine in a humid atmosphere with 5% CO_2 at 37°C. All medium, supplements, and sera were purchased from Invitrogen (Grand Island, NY, USA). Cultures were passaged by dilution to a cell concentration of 2×10^5/ml to maintain asynchronous and exponential growth.

Cell synchronization

A double-thymidine block was used to arrest HeLa cells at G1/S transition (31). A stock solution of 100 mM thymidine (Sigma Chemical Co., St. Louis, MO, USA) was made by dissolving thymidine in media without serum and stored at 4°C. HeLa cells were cultured in RPMI-1640 supplemented with 10% FBS. HeLa cells were first synchronized by the addition of 2 mM thymidine from a 100 mM stock solution for 18 h. Cells were then washed twice with phosphate buffer saline (PBS) followed by the addition of regular culture media. Then, 9 h after the release, the media was changed to that containing 2 mM thymidine for another 17 h. Cells were collected by 0.01% trypsin-EDTA every two hours after they were released from the second thymidine block. Trypsin was neutralized by adding medium containing 10% FBS. To determine the time point at which the majority of the synchronized cells were in G1, S or G2/M phase, cells were collected at different intervals and fixed in 70% ethanol and analyzed by propidium iodide (PI) staining and flow cytometry.

Flow cytometry

Cells were washed once in PBS, and fixed in 70% ethanol overnight at 4°C. Staining for DNA content was performed with 50 mg/ml PI and 1 mg/ml RNase A for 30 min at 37°C in the dark prior to flow cytometry analysis (32). Analysis was performed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with CellQuest Pro software. Cell-cycle modeling was performed with Modfit 3.0 software (Verity Software House, Topsham, ME, USA). For each sample, 10,000 events were collected and aggregated cells were gated out. Percentages of cells existing within the different phases of the cell cycle were calculated using CellQuest by gating on G1, S and G2+M cell populations.

RNA extraction and quality control

Total RNA was extracted from HeLa harvested after 4 h, 10 h, and 16 h after release from the second thymidine block using a mirVana miRNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturers' instructions. The integrity and stability of RNA samples were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

miRNA microarray analysis

A poly (A) tail was appended to the 3'-end of enriched miRNAs from all the above samples with a mixture of unmodified and amine-modified nucleotides (Ambion). The tailed samples were fluorescently labeled using an amine-reactive Cy3 dye (Amersham), and the unincorporated dyes were removed with glass fiber filters. The samples were hybridized for 18 hr onto slides arrayed with miRNA probes from the NCode™ human miRNA Microarray Probe Set (Invitrogen). Slides were then washed 3 × 2 min in 2 × SSC and scanned using a Generation III array scanner (Amersham Pharmacia).

Bioinformatics analysis

Raw miRNA expression data were preprocessed stepwise: background signals were subtracted from foreground signals followed by robust calibration and variance stabilization (33); Values of duplicated probes were averaged and data for miRNAs containing "hsa-" in their labels were retained. This produced a list of 698 unique miRNAs. Differentially expressed miRNAs showing cell-cycle-dependent patterns were enriched by "Multiclass" testing in SAM or test-fitting with lmFit/eBayes as suggested by the R "limma" package (34). In addition, SOM (35) was used to classify 25 differential miRNAs into five groups based on their cell cycle-dependent expression patterns.

qRT-PCR analysis for miRNAs expression

Total RNA with good quality was used for the following analysis. The miRNA sequence-specific primers for hsa-let-7a (Cat# AM30000), hsa-miR-24 (Cat# AM30121), hsa-miR-376b (Cat# AM30269), hsa-miR-21 (Cat# AM30102), hsa-miR-221 (Cat# AM30115), hsa-miR-34a (Cat# AM30168) and endo-

genous control 5S rRNA (Cat# AM30302) were purchased from Ambion. Real-time qRT-PCR analysis was carried out on a Stratagene MX3000P instrument with mirVana qRT-PCR miRNA detection kit (Ambion, Cat #AM1558, USA) and amplified product levels were detected by real-time monitoring of SYBR Green I dye fluorescence under the following conditions: 37°C, 30 minutes; 95°C, 10 minutes of reverse transcription; 95°C, 3 minutes; 40 cycles of 95°C, 15 seconds, 60°C, 30 seconds for the amplification. Each PCR reaction was done in triplicate.

The relative quantification values for each miRNA were calculated by the $2^{-\Delta\Delta C_T}$ method (36, 37) using 5S rRNA as an internal reference. The level of expression at S and G2/M for each miRNA is presented relative to its expression at G1 after normalizing for 5S rRNA. The ΔC_T was calculated by subtracting the C_T of 5S rRNA from the C_T of the miRNA of interest. The $\Delta\Delta C_T$ was calculated by subtracting the ΔC_T of the reference sample from the ΔC_T of each sample. Fold change was calculated using the equation $2^{-\Delta\Delta C_T}$.

Statistical analysis

Data were presented as Mean ± SD and independent t test analysis was performed by SPSS13.0 software to compare the difference between different phases. A value of $P < 0.05$ was considered statistically significant.

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