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Studying the Role of miR-141 in Supporting Cervical Cancer Cell Proliferation

Emad Dabous[1], Adel, A. Guirgis[1], Hany Khalil[1*]

[1]Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt

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***Corresponding author:** Hany Khalil
Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City, Egypt

Abstract

MicroRNAs (miRNAs) are small noncoding RNA, approximately 18-23 nucleotides that can post-transcriptionally regulate the expression of complementary mRNAs. MiRNAs have been found to play a critical role in a broad spectrum of biological processes, such as developmental timing, cell death, cell proliferation, hematopoiesis, and nervous system patterning. Here, we aimed to investigate the possible upregulation of miR-141 in cervical cancer cells and to confirm the influential role of miR-141 in cervical cancer cell proliferation. The level of miR-141 in HeLa cells has been assessed using quantitative real-time PCR (qRT-PCR). Cell morphology and a number of living HeLa cells were achieved upon transfection with either precursor miR-141 (pre-miR-141) or a specific inhibitor. MTT assay and lactate dehydrogenase (LDH) production were monitored to assess the potential toxic effect of miR-141 in cancer cells. ELISA assay has been used to monitor the produced cytokines from transfected HeLa cells. Notably, the expression of miR-141 significantly increased in HeLa cells compared to the normal cervical HCK1T cell line. Transfection of HeLa cells with an inhibitor, antagonist miR-141, showed a potent effect on cancer cell viability, unlike the transfection of pre-miR-141. HeLa cells transfected with pre-miR-141 showed decreased levels of interleukin 13 (IL-13). Meanwhile, the transfection of miR-141 specific inhibitor showed an increasing level of produced IL-10 and a decreasing level of IL-10, indicating the role of miR-141 in avoiding programmed cell death in HeLa cells. Together, these data uncover the role of miR-141 in supporting cervical cancer progression and provide miR-141 as a believable therapeutic target.

Keywords: miR-141; Cervical cancer; Cell proliferation; IL-8, IL-10.

INTRODUCTION

Cervical cancer is a public health problem and the fourth most common cancer among women worldwide. About 569,847 new cases and 311,365 deaths have been registered annually. A systematic study reported that by 2030, the number of cervical cancer cases will increase by around 50% worldwide (Ferlay *et al.*, 2018)**.** In recent decades**,** the mortality and morbidity rate of cervical cancer has decreased in several countries due to effective prevention programs and successful screening programs (Arbyn *et al*., 2020)**.** However, in developing countries, the screening programs recognize only the women test in primary health care centers more than in clinical health centers. Accordingly, the current treatment programs in these countries lose the significant impact on reducing mortality and morbidity rates of cervical cancer (Hull1 *et al*., 2020). Usually, cancer occurs through various stages in which cellular factors control each step. For example, the expression of epidermal growth factor receptor (EGFR), KRAS, BRAF, and depletion in tumor suppressor factors such as P53 and PTEN have been associated with cancer initiation (Kamiya *et al*., 2019; Balcik-Ercin *et al*., 2020). Vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α), and epidermal growth factor (EGF) are critical angiogenic factors (Hoeben *et al*., 2004). Noteworthy, microRNAs (miRNAs) are small noncoding RNA; approximately 18-23 nucleotides that can post-transcriptionally regulate the expression of its cleaved messenger RNAs (mRNAs). Hundreds of miRNAs genes have been identified in diverse animal and many of phylogenetically conserved species (Kaufman and Miska, 2010). In mammalian cells, miRNAs are up-regulated upon several microbial infections to modulate

a variety of intracellular signaling. Other cumulative evidences indicated that some endogenous miRNAs are able to interact as a tumor suppressor or oncogenes during cancer development. For instance, miR-21 is constantly up-regulated in gastric cells caused by *Helicobacter pylori* infection leading to colorectal cancer disorder (Volinia *et al.*, 2006). The direct targets of miR-21 include tumor suppressor protein tropomyosin 1 (TPM1) and metastasis suppressor reversion-inducing-cysteine-rich protein (RECK) (Zhu *et al.*, 2008). Evidence reported the efficacy of miRNAs in cervical cancer development and resistance to drugs. (Abbas *et al*., 2021) For instance, miR-126 has been reported in cervical cancer diagnosis, while high level of miR-143 or inhibition of miR-21 has been incorporated into cervical cancer therapy (Banno *et al*., 2014). Accordingly, we investigated the possible upregulation of miR-141 in cervical cancer cells and identified its role in cell proliferation.

MATERIAL AND METHODS

Cell Lines

The human cervical cancer HeLa cell line and the normal cervical keratinocytes cell, HCK1T cell line, were obtained from (VACSERA, Giza, Egypt) and were cultured in Roswell Park Memorial Institute (RPMI) 1640 media, containing 25 mM HEPS, 4 mM L-glutamine, and 10% of heat-inactivated bovine serum albumin (BSA) and incubated at 37°C and relative humidity of 95% (El-Fadl *et al*., 2021).

Transfection protocol

HeLa cells were overnight cultured in 6-well plates with confluency of about 80%. HeLa cells were then transfected with either a respective inhibitor antagonist miR-141 (5-′ACAACCACTGTCTGGTAAAG-3′) or pre-miR-141 using Lipofectamine LTX (Invitrogen, USA). According to the manufacturer's instructions, the cells were transfected with 12.5 µg/ml using 20µl Lipofectamine LTX prepared in 500 µl optimum media. Cells transfected with the same concentration of transfection reagents were severed as control-transfected cells. The knockdown efficiency of miR-141 and the relative expression of indicated genes were monitored in transfected and infected cells using qRT-PCR. Flow cytometry assay was used to detect the kinetic protein expression of MxA and STAT3 in transfected and infected cells (Abd El Maksoud *et al*., 2020).

Proliferation and cytotoxic assay

For proliferation assay of transfected HeLa cells with either miR-141 inhibitor or precursor, cells were cultured in duplicate in a 6-well plate at $10X10^4$ cells per well. Cell morphology was monitored using the inverted microscope. The number of survived cells upon transfection was accounted by using a hemocytometer. In brief, the old media was discarded, and the cells were washed twice with phosphate buffer saline (PBS) before trypsinizing by 3 min incubation at 37ºC. Then a suitable volume of complete RPMI media was added to the trypsinized cells, and the number of cells was manually accounted (Hamouda *et al*., 2021; Mohamed *et al*., 2022). To investigate the cytotoxicity of miR-141 transfection, HeLa cells were seeded in triplicate in 96-well plates at $10x10^3$ cells per well and were incubated overnight. Cells were transfected with varying concentrations of pre-miR-141 or specific inhibitor (12.5-200 µg/well). Cells treated with transfection reagents served as control-transfected cells. The cell viability rate was monitored by using MTT colorimetric assay kit (Sigma-Aldrich, Germany). In brief, the cultured media was discarded, and PBS was used for washing the cells; then 100 µl RPMI media was added to each well. Next, 10 µl MTT solution was added, and the plate was incubated for 1 hour at 37ºC. Finally, 100 µl SDS-HCl was added to each well in the plate which then was incubated for 4 hrs at 37ºC. Cell viability was monitored depending on the amount of converted water-soluble MTT to an insoluble formazan which is then solubilized and determined by optical density at 570 nm.

Detection of miR-141 relative expression

To detect the expression level of miR-141, small miRNAs was extracted from transfected HeLa cells (48 hours post-transfection) using PureLink™ miRNA Isolation Kit (Invitrogen, USA) according to the manufacturer's protocol. RT-PCR was used to detect the relative expression of miR-141 using specific primers listed in Table 1. First, cDNA was performed from small miRNAs using reverse transcriptase reaction followed by the amplification step using miR-141 and RNU6B specific TaqMan microRNA assays (Applied Biosystem, Darmstadt, Germany), according to the manufacturer's protocol. Levels of RNU6B were used for normalization. To perform cDNA from small miR-141, the following reagents were prepared for reaction: 0.15 µl dNTPs (100 mM), 1 µl reverse transcriptase (50 U/µl), 1.5 µl 10X reverse transcriptase buffer, 0.2 µl RNase inhibitor (20 U/µl), 5 µl purified miRNAs (10 ng/µl) and 1 µl from each primer up to final volume of 20 µl using RNase free water. The mixture then incubated 30 min at 42°C followed by 5 min at 85°C to inactivate the enzyme. The resulting cDNA then was used as a template to amplify both miR-141 and RNU6B by using the following parameters in quantitative real-time PCR (qRT-PCR) machine: 95 ˚C for 5 min, 40 cycles (95 ˚C for 15 sec, 60˚C for 15 sec and 72°C for 15 sec) and 72°C for 3 min.

Table 1: Oligonucleotides sequences used for quantifying miR-141 in transfected HeLa cells

Description	Primer sequences 5'-3'
MiR-141-sense	CGCTAACACTGTCTGGTAAAG
MiR-141 antisense	GTGCAGGGTCCGAGGT
MiR-U6-sense	GCTTCGGCAGCACATATACTAAAAT
MiR-U6-antisense	CGCTTCACGAATTTGCGTGTCAT

Elisa assay

For the quantification of the released IL-10 and IL-13, ELISA assay was performed using human ELISA kits (Abcam 46043 and Abcam 288591, respectively). Consequently, HeLa cells were overnight cultured in a 96-well plate with a density of 10000 cells/well. Afterwards, the cells were transfected using a 20µl optimum medium which contains 25 µg/well of either miR-141 inhibitor or precursor suspended in 2 µl HyperFect. Transfected cells were then incubated for 6 hours; then, a fresh RPMI medium was added to each well instead of the transfection medium, and cells were incubated for two days. Finally, both treated and transfected cells were incubated for different periods ranging from 0, 6, 12, 24, and 48 hrs. At each time, 50 µl of the lysed cells were transferred into the ELISA plate and incubated for 3 hrs at RT with the volume similar to the control solution and 1X biotinylated antibody. Following washing, 100µl of 1X streptavidin-HRP solution was added to each well which then was incubated for 30 min in the dark. Then, 100 µl of the chromogen TMB substrate solution was added to each well, followed by 15 min incubation at RT away from the light. The stop solution was added, and the absorbance of each well was monitored using 450 nm (Alalem *et al*., 2023).

Data analysis

For quantification the cycle threshold (Ct) of each investigated gene expression, delta-delta-Ct equations were used as previously described: (1) delta-Ct = Ct value for gene - Ct value for GAPDH, (2) (delta–delta-Ct) = delta-Ct for experimental–delta-Ct for control, (3) relative expression of targeted gene = ($2^{-\text{delta-delta ct}}$) (Rao *et al*., 2013; Khalil H *et al*., 2017) .Statistical analysis was done using the student's t-test between two groubs. *P*-value $\leq$ 0.05 was considered statistically significant.

RESULTS

miR-141 is upregulated in HeLa cell and regulates cell proliferation

The expression level of miR-141 was first achieved in different cervical cancer cells, including HeLa cells, compared to its expression level in HCK1T cells. As shown in Figure 1A, the expression miR-141 significantly upregulated in the cervical cancer cell line with more than 10-fold change compared with the normal HCK1T cells. To determine whether miR-141 plays any role in HeLa cell proliferation, the transfected cells with either pre-miR-141 or inhibitor antagonist miR-141 were investigated. Unlike transfection with pre-miR-141, the transfection with miR-141 specific inhibitor showed a marked alteration of cell morphology compared to control-transfected cells, evidenced by spaced cells (Figure 1B). Furthermore, the number of living HeLa cells transfected with the inhibitor antagonist miR-141 was significantly reduced compared with control-transfected cells and untreated cells (Figure 1C). These data demonstrate that the expression level miR-141 is upregulated in HeLa cells to support cancer cell proliferation, indicating the supportive role of miR-141 in cancer development.

Antagonist miR-141 effectively regulates HeLa cell proliferation

Cell viability and produced LDH from transfected cells have been monitored further to confirm the role of miR-141 in cervical cancer cells. Interestingly, the cell viability rate of HeLa cells transfected with miR-141 inhibitor strongly decreased in dose-dependent inhibitor concentrations (Figure 2A). In contradiction, cells transfected with pre-miR-141 showed neglected differentiation in cell viability rate compared with control-transfected cells and an increasing rate compared to untreated cells (Figure 2A). Likewise, LDH production significantly increased in HeLa cells transfected with the respective inhibitor antagonist miR-141 compared with its level in cells transfected with pre-miR-141 and control-transfected cells (Figure 2B). These data demonstrate the potential regulatory role of miR-141 in regulating HeLa cell proliferation and the influential part of targeting miR-141 in inducing programmed cell death in cancer cells.

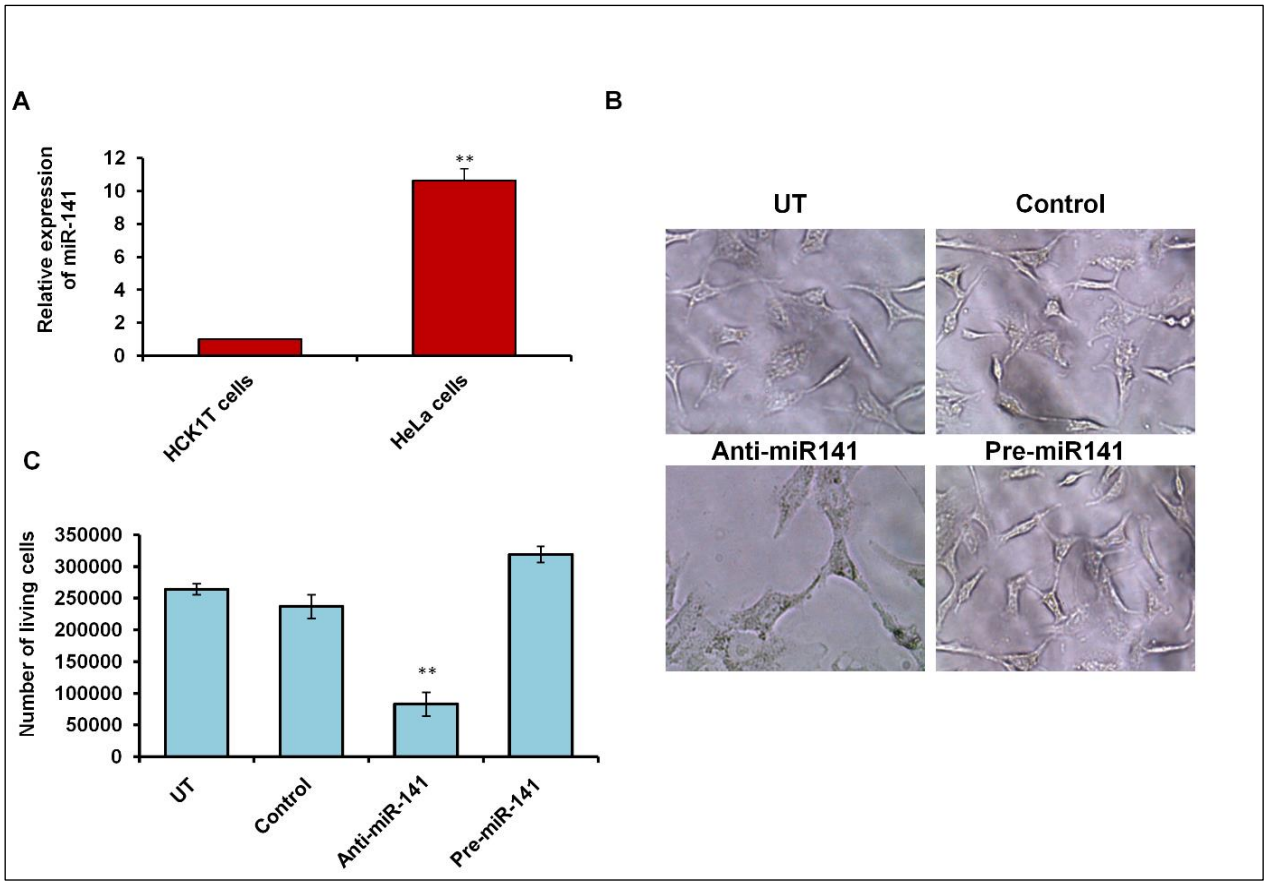

Figure 1: The role of miR-141 in cervical cancer cell proliferation; (A) Quantification of miR-141expression level in cervical cancer HeLa cells compared with the normal cervical HCK1T cells indicated by fold changes using qRT-PCR. (B) HeLa cell morphology indicated by inverted microscope upon 48 hrs of transfection with either pre-miR-141 or an inhibitor antagonist miR-141 compared with control-transfected and untreated cells. (C) After transfection, the number of living HeLa cells. Error panels indicate the standard deviation (STD) of three independent experiments. Student two-tailed *t*-test used for statistical analysis, (*) indicates *P-values* ≤0.05, (**) indicates *P* ≤0.01, and (***) indicates *P* ≤0.001

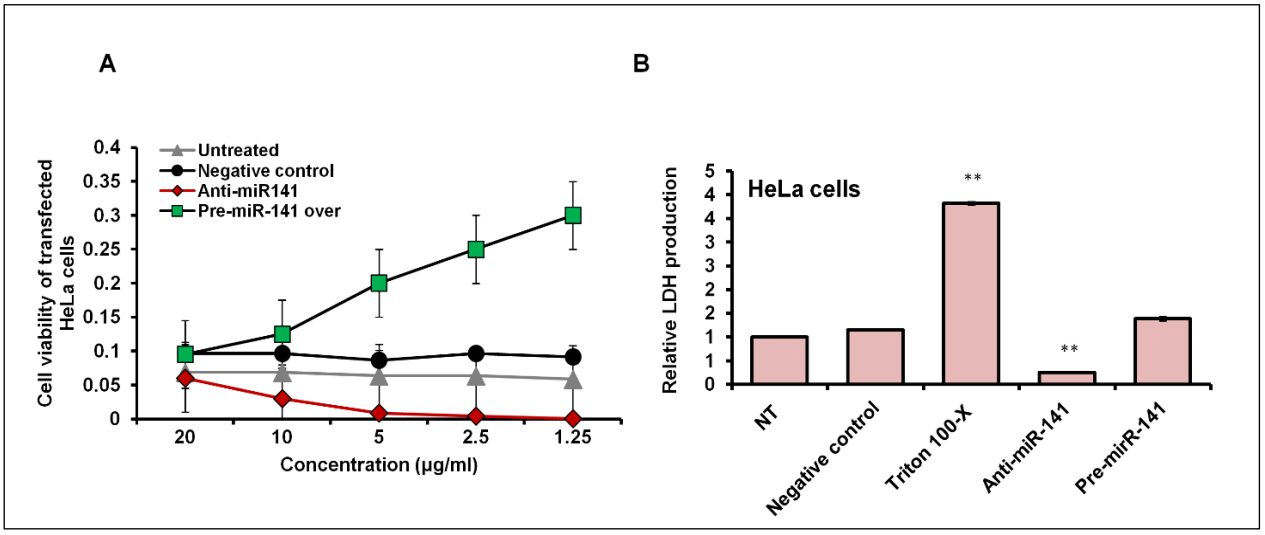

Figure 2: Cytotoxic influence of pre-miR-141 and specific inhibitor transfection in HeLa cells; (A) Cell viability rate of transfected HeLa cells with different concentrations of the pre-miR-141 and specific inhibitor indicated by the absorbance rate of treated cells with MTT agent. (B) The relative LDH produced in the fluid media of transfected HeLa cells compared with cells treated with Triton 100-X as negative control and nontreated cells (NT). Error panels present the STD of three independent experiments. Student two-tailed *t*-test was used for statistical analysis

Deception of miR-141suessfully modifies the production of IL-10 and IL-13 in transfected HeLa cells

In a time-course experiment, the concentration of secreted IL-10 and IL-13 was monitored in transfected HeLa cells. The level of produced IL-10 significantly reduced in cells transfected with pre-miR-141 while markedly increasing in cells transfected with the inhibitor antagonist miR-141 in a similar level to control-transfected cells (Figure 3A). IL-10 showed little differentiation in transfected HeLa cells compared with non-transfected (NT) and control-transfected cells (Figure 3A). In contrast, the amount of produced IL-13 increased in cells transfected with miR-141 specific inhibitor in a time-dependent manner and reached 500pm/ml at 48 hrs post-transfection. While the level of IL-13 markedly decreased in cells transfected with a pre-miR-141 compared with control transfected cells. These data indicate that inhibition of miR-141 expression increases the produced IL-13 in HeLa cells without affecting the anti-inflammatory cytokine IL-10.

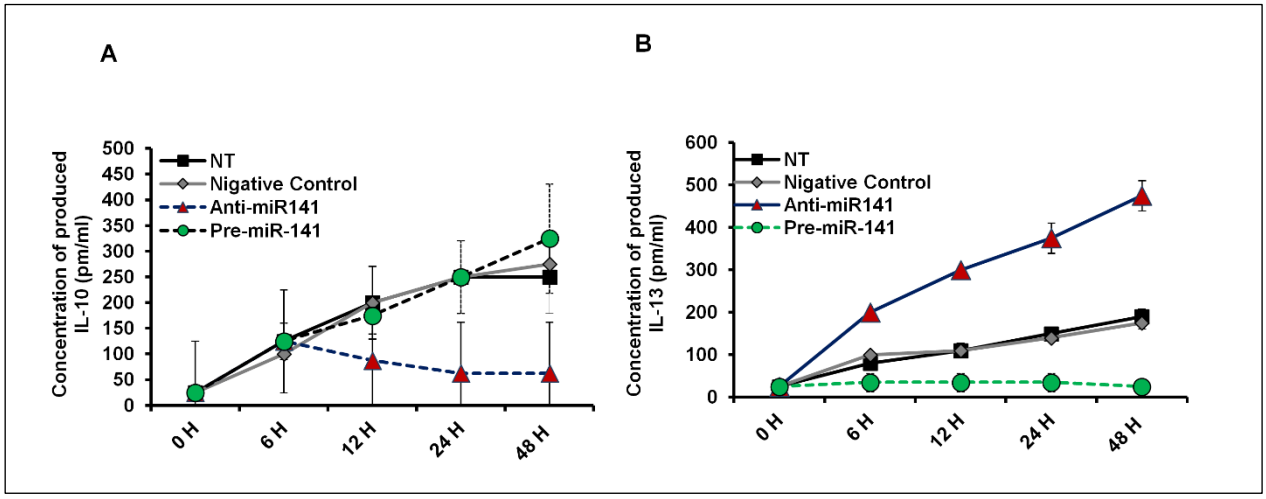

Figure 3: Levels of produced inflammatory cytokines in transfected HeLa cells, (A) The concentration of IL-10 (pm/ml) produced in the fluid media of transfected HeLa cells in response to the expression level of miR-141 at the indicated time points. (B) The concentration of IL-13 in the culture media of transfected HeLa cells simultaneously post-transfection

DISCUSSION

The biogenesis pathway of miRNAs includes the incorporation of mature miRNA with RNA-induced silencing complex (RISC) in which miRNA can post-transcriptionally regulate targeted gene expression. The miR-200 family of microRNAs (miRNAs) includes miR-200a, miR-200b, miR-200c, miR-141, and miR-429. Five evolutionarily conserved miRNAs that are encoded in two clusters of hairpin precursors located on human chromosome 1 (miR-200b, miR-200a, and miR-429) and chromosome 12 (miR-200c and miR-141). The mature -3p products of the precursors increase in epithelial cells, which implicates maintaining the epithelial phenotype by suppressing the expression of the vital hallmark factors of oncogenic transformation that favor the process of epithelial-to-mesenchymal transition (EMT). Recent study of the expression and interactions of these miRNAs with cell signaling pathways indicate that they can exert both tumor suppressor- and pro-metastatic functions and may serve as biomarkers of epithelial cancers (Cavallari *et al*., 2021). In this way, we investigated the expression level of miR-141 in cervical cancer cell lines, which was upregulated in HeLa cells compared with the normal cervical cells. Noteworthy, increasing evidence has demonstrated abnormal miRNA profiles and confirmed their involvement in tumor initiation and progression. As a critical member of the miR-200 family, miR-141 is aberrantly expressed in many human malignant tumors, participating in various cellular processes, including epithelial-mesenchymal transition (EMT), proliferation, migration, invasion, and drug resistance (Gao *et al*., 2016). Other miRNAs also implicated in cancer progress such as miR-21. Upregulation of miR-21 in colorectal cancer obtained samples results in direct interaction and targeting so many genes such as STAT3, PPARA, TGF-β, and PDCD4 as predicted in a recent study (Maher *et al*., 2020). Importantly, STAT3 belongs to STAT protein family that implicated in cancer development through its regulatory role in cell proliferation, malignancy transformation, tumor angiogenesis and metastasis (Levy and Lee, 2002). In colon cancer, miR-141 has been identified as a supportive factor for cell proliferation via targeting PH domain leucine-rich-repeats protein phosphatase 2 (*PHLPP2)* in vivo and in vitro study. In addition, circulating miR-141 was recognized as a potential biomarker for diagnosis, prognosis and therapeutic targets in gallbladder cancer (Yang *et al.*, 2022). Likewise, miR-141-3p regulates proliferation, chemosensitivity, migration and invasion of colorectal cancer cells by targeting EGFR (Xing *et al.*, 2020). Notably, the role of miR-141 in regulating interleukins production has been observed. For instance, a CRISPR/Cas9-based method targeting miR-141 in primary human bronchial epithelial cells at air-liquid-interface and goblet cell hyperplasia induced by IL-13

stimulation. miR-141 disruption decreased goblet cell frequency, intracellular MUC5AC, and total secreted mucus. These effects correlated with a reduction in a cellular gene expression signature and enrichment of a basal cell gene expression signature defined by single-cell RNA sequencing (Siddiqui *et al*., 2021). Importantly, here we confirmed the possible regulatory role of miR-141 in regulating IL-6 production in cancer, evident by increasing level when targeting miR-141 by specific inhibitor that resulted in programmed cell death. Interestingly, several studies reported the regulatory role IL-13 in apoptosis with a delay of necrotic event such as a study demonstrated that IL-13 alleviated sepsis-induced apoptosis by improving mitochondrial fatty acid and oxidation (Guo *et al.*, 2021).

CONCLUSION

The present study shows the upregulation of miR-141 in cervical cancer HeLa cells compared to the normal cervical HCK1T cells. The inhibition of miR-141 by a respective inhibitor showed a potent disturbance of cell viability and cell morphology accompanied by a significantly produced level of LDH, indicating the role of miR-141 in supporting cell proliferation. The increasing level of IL-13 in HeLa cells transfected with miR-141 inhibitor suggests the role of miR-141 in avoiding apoptotic signaling cervical cancer cells.

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Authors' Contributions

Emad Dabous, performed the experiments. Adel Guirgis helped in supervision and conceptualizing experiments. Hany Khalil designed the research plan, supervised overall research, provided and interpreted data, organized, and wrote the manuscript.

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