

# Concurrent Targeting of MEG3 and Linc-ROR Increases the p53 Transcript in HCT116 Cells

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## Research article

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# Abstract

Recently, lncRNAs are used as a prospective strategy in cancer gene therapy. Dysregulation of the maternally expressed gene3 (MEG3) and the linc-ROR have demonstrated in colorectal cancer researches. This study evaluates the effect of concurrent silencing of linc-ROR and MEG3 activation on colon cancer cell survival in order to assess the p53 transcriptional activity and stability.

The MEG3 and linc-ROR shRNA were cloned under the bidirectional CEA promoter (UM1). Transfection efficiency was examined by GFP expression under fluorescent microscope. Following transfection, the response of HCT116 cells was evaluated by MTS assay, apoptosis and cell cycle analyses. The expression of target genes along with the p53 were analyzed in the transcription level by qPCR.

Proliferation of both cancer cell lines were significantly reduced at 48 h post-transfection. The UM1 significantly induced apoptosis in HCT116 (36.35%). Moreover, UM1 remarkably reduced the cell population in S phase. Also, concomitant up- and down-regulation of MEG3 and linc-ROR were attributed to the activation of p53 in transcription level.

Concurrent silencing of linc-ROR and MEG3 activation in colon cancer cells resulted in the activation of p53 and reduced the cell proliferation. We found that the higher apoptosis induction was coupled with the enhancement of the p53 expression. The synergistic effect of target genes might serve as a useful approach for targeting p53 in colon cancer.

# Introduction

Colorectal cancer (CRC) is already the third common cause of cancer in terms of incidence and the second most deadly cancer in the world. Due to the changes in lifestyle and everyday diet, the incidence of CRC has been increased. CRC is a complex and molecularly heterogeneous disease (1, 2). Multiple genetic and epigenetic alterations occur in the transition from normal colonic mucosa to invasive adenocarcinomas (3). Despite advances in diagnosis and treatment of CRC, the recurrence and mortality still remain high, with 5-year survival rate less than 65% (4–6).

Long non-coding RNAs (lncRNAs) are defined as RNA transcripts with more than 200 nucleotides in length, while do not harbors any open reading frame, and thus have no protein-coding potential. LncRNAs are expressed in many loci of the genome and modulate gene expression in the nucleus and the cytoplasm (7, 8). They are involved in a large range of biological processes as gene-regulatory components. Also, the oncogenic or tumor suppressor roles of these molecules have been demonstrated (9). Moreover emerging studies have revealed that aberrant/dysregulated expression of lncRNAs have linked to the pathogenesis of cancer (10, 11). LncRNAs may be involved in carcinogenesis and the progression of tumors through a variety of mechanisms by interfering in diverse cell signaling pathways (12–14).

Due to their critical functions in cancer development, they have been attracted as the potential therapeutic targets for cancer gene therapy (11, 15).

One such lncRNA gene is maternally expressed gene 3 -MEG3 (also known as gene trap locus 2 (GTL2)), located in chromosome 14q32. MEG3 RNA is expressed in many normal tissues, while aberrantly expressed in multiple cancers such as cervical cancer, prostate cancer, bladder cancer, and CRC (16–19). MEG3 has been found as tumor suppressor and inhibits cancer cell proliferation, migration, and induce apoptosis *in vitro* and *in vivo* by stimulating the p53-dependent transcription (20, 21).

Moreover, lincRNA-Regulator of Reprogramming (linc-ROR) is located at chromosome 18q21.31. It was initially reported as an important modulator in the reprogramming differentiated cells to induced pluripotent stem cells (iPSCs) (22). Growing evidence demonstrated that its aberrant expression and oncogenic role in many types of malignant carcinomas including breast cancer, pancreatic cancer, gallbladder cancer nasopharyngeal carcinoma and CRC (23–25).

Further research elucidated that the linc-ROR could negatively regulate the p53 and act as inhibiting the p53-mediated cell cycle and apoptosis (23, 26). Therefore, a strategy to overcome the resistance to p53-dependent apoptosis is to target the regulators that influenced the expression of the p53. Here, we aimed to explore the simultaneous effect of linc-ROR silencing and increasing expression of MEG3 on the survival of HCT116 cells. The cell response to the transcription expression level of p53 in response to this recombinant vector was evaluated.

## Material And Methods

### Plasmid construction

The full length of MEG3 sequence along with the sequence of linc-ROR shRNA was synthesized by Biomatik Company (Canada) and subcloned into the pRNAT-U6.1/Neo vector harboring the fluorescent marker, EGFP. The expression of target genes were controlled by a bidirectional CEA promoter and so called UM1.

### Cell culture and cell transfection

Human CRC cell lines (HCT-116) were obtained from Pasteur Institute (Tehran, IRAN). Cells were cultured in the DMEM-F12 medium (Bioidea, Iran) supplemented with 10% fetal bovine serum and penicillin/streptomycin antibiotic (1%; Bioidea, Iran). They were incubated at 37 °C in humidified air with 5% CO<sub>2</sub>.

The cells were transfected in the 6-well plates (10<sup>6</sup> cells/well) using Lipofectamine3000 (Life Technologies, USA) according to the manufacturer's instructions.

Two experimental groups were designed in the current study including (1) bidirectional vector containing the MEG3 sequence and the linc-ROR shRNA (UM1) and (2) empty vector, pRNAT-U6.1 as a control group.

# Transfection efficiency assay

In order to assess the transfection efficiency, GFP signals were considered. Cells ( $2 \times 10^5$  cells/well) were monitored under fluorescent microscope following 24 and 48 h post-transfection with the UM1.

## Cell viability assay

Cells were seeded into 96-well plates and transfected with the UM1 vector at 24, 48, and 72 hours. The viability of transfected cells were then assessed by MTS solution (Promega, USA) based on the manufacturer's protocol. The absorbance was measured at 490 nm by ELISA reader (Biotek, Germany).

## RNA extraction, cDNA synthesis and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from cultured cells with RiboEX reagent (GeneAll, Korea) according to the manufacturer's protocol. 1  $\mu$ g of total RNA was reverse transcribed to obtain cDNA using reverse transcription kit (yekta tajhiz azma, Iran). The QRT-PCR was performed with SYBR green real-time Master Mix (yekta tajhiz azma, Iran) by the LineGene K system. Beta actin was used as the internal control. The characteristics of primers were presented in Table 1. The relative expression was calculated using the  $2^{-\Delta\Delta Ct}$  method and normalized to the expression of Beta actin.

## Apoptosis detection

The HCT116 cells were cultured in the 6-well plate and harvested at 48 hours after transfection. Apoptosis was measured using an FITC Annexin V Apoptosis Detection Kit with PI (bioligand; USA) accordance with the manufacturer's protocols. Briefly, cells were resuspended in the Annexin V Binding Buffer and double-stained with 5  $\mu$ l Annexin V-FITC and 10  $\mu$ l propidium iodide (PI). Subsequently, the solution was incubated in the dark for 15 min. Finally 400  $\mu$ l binding buffer was added, then the stained cells were analyzed by flow cytometer equipped with BD Accuri™ C6 plus Cell software. Cells were discriminated into viable cells, dead cells, early apoptotic cells, and late apoptotic cells. The percentages of apoptotic cells were compared with non-transfected cells as control by the FCS Express 7 software.

## Cell cycle analysis

Following 48 h post-transfection, the transfected cells were harvested and fixed in 70% ethanol at  $-20^\circ\text{C}$  for 2 hours. Fixed cells were washed with PBS and incubated in PBS containing 100  $\mu\text{g/ml}$  RNase A and incubated at  $37^\circ\text{C}$  for 30 min to eliminate the intracellular RNA. Finally, cells were stained with propidium iodide (PI) (10  $\mu\text{g/ml}$ ) in the dark for 30 min at room temperature, followed by flow cytometry analysis. The percentages of the cell population in G<sub>0</sub>/G<sub>1</sub>, S, or G<sub>2</sub>/M phase were determined and compared with the control group by the FlowJo software.

## Statistical analysis

Data were analyzed using SPSS 17.0 software. All data were expressed as the mean  $\pm$  SD. Significance values were determined by student's t-test.

## Results

### Construction of UM1

Figure 1a illustrates the map of UM1 vector (9577 bp). The expression of MEG3 and linc-ROR shRNA are controlled by the bidirectional CEA promoter. The resultant construct was verified by double digestion assay (Fig. 1b) and DNA sequencing.

### UM1 vector inhibits cell proliferation

The efficiency of transfection was monitored through the expression of GFP by the fluorescent microscope to optimize the best transfection time. The maximum expression of GFP was observed at 48 h post-transfection (Fig. 2a). The proliferation of HCT116 cells in response to UM1 vector was examined by the MTS assay. The lowest proliferative ability was observed in the UM1 transfected cells compared with the negative control group (U6) following 48 h post-transfection (Fig. 2b). The UM1 expression in the HCT116 cells was significantly reduced the cell growth ( $p < 0.05$ ).

### MEG3 and linc-ROR expression regulated by UM1

To investigate the functional role of bidirectional UM1 vector in colorectal cancer cells, we explored the effect of simultaneous the MEG3 over-expression and silencing of linc-ROR in the HCT116 cell line. Figure 3 shows the relative transcription level of MEG3, linc-ROR shRNA, and the p53 after 48 h transfection.

The MEG3 expression was remarkably up-regulated in the transfected cells than the control ( $p < 0.05$ ). Whilst, the linc-ROR expression was significantly reduced ( $p < 0.05$ ), indicated that the bidirectional vector has affected on the target lincRNAs. As shown in the above image, the MEG3 expression was increased 642-fold in the HCT116 transfected cells whereas the linc-ROR expression was reduced 0.13-fold in the same cells. The expression level of p53 was also up-regulated (1.54-fold) in response to the MEG3 overexpression and silencing of the linc-ROR ( $p < 0.05$ ).

### Combinatorial effects of induced MEG3 and linc-ROR shRNA on apoptosis

To determine whether simultaneous targeting lincRNAs lead to apoptosis induction and influenced the cell cycle distribution *in vitro*, Annexin V/PI staining protocol was performed and analyzed by the Flow cytometry. The results indicated that the apoptotic rates of the UM1 transfected cells was significantly increased compared to the control (Fig. 4a). Moreover, the cell cycle analysis was conducted by DNA content measurement in the transfected HCT116 cells and non-transfected cells as control (Fig. 4b). The G1 phase in the UM1 transfected cells was significantly increased from 52.7% (NC) to 81.5% ( $p < 0.01$ ). Also, S and G2/M phase percentages were decreased obviously from 25.1% (NC) to 7.8% ( $p < 0.05$ ) and from 18.1% (NC) to 10.7% ( $p < 0.05$ ), respectively. These findings indicate that UM1 can induce G1/S arrest and drive the late apoptosis, which may lead to the inhibition of the cell proliferation.

## Discussion

A relationship between the dysregulation of lncRNAs and cancer development and progression has been reported in the literature (27, 28). Recently, few lncRNAs have been identified as a biomarker in the colorectal cancer (15, 29). However, the functional impact of lncRNAs in the colorectal cancer is still largely unknown (27, 30). Therefore, this study set out with the aim of assessing the importance of MEG3 and linc-ROR on the survival of the HCT116 cells at the same time. The current study found that the simultaneous induction of MEG3 and silencing of linc-ROR resulted to the reduction of cell proliferation. This finding matches those observed in the prior studies. As previously demonstrated, the MEG3 gene acts as a tumor suppressor (31) and its activation inhibits the proliferation of prostate cancer cells (32). The same results were observed in the breast and gastric cancers (20, 21). Moreover, the high throughput data revealed that the MEG3 expression was remarkably reduced in the colon adenocarcinoma (33). However, no data was found regarding to the linc-ROR expression in the same cancer type. In contrast to the findings attributed to the investigations on the linc-ROR silencing (34–36), however, no evidence of the linc-ROR expression in the colon adenocarcinoma in the GEPIA database was detected.

Our results showed that the MEG3 activation and the linc-ROR silencing reduced the cell proliferation and up-regulated the p53 expression in transcript level in the HCT116 cells. Interestingly, the most remarkable reduction in the cell proliferation was occurred at 48 h post-transfection ( $p < 0.01$ ); at the same time period that the expression of MEG3 and the linc-ROR significantly were altered. Increased activation in the p53 in this study corroborates the earlier researches, who suggested that linc-ROR is a repressor of p53 in response to DNA damage (34). They introduced this lncRNA as a strong negative regulator of p53. They revealed that the linc-ROR by hnRNP I and the MDM2 through the ubiquitin-proteasome pathway resulted in the reduction of p53 protein in the MCF-7 and the HCT116 cells. Another research group showed that the linc-ROR inhibited the p53 expression and affected the p53 target genes in various colorectal cancer cell lines (35). Additionally, the linc-ROR suppresses the chemotherapy resistance ability of the nasopharyngeal carcinoma by the p53 pathway (37). The results of this study indicates that knockdown of linc-ROR caused the activation of p53 pathways. They also introduced this lncRNA as a negative regulator of p53.

These findings further support the idea of both lncRNAs involvement into the pathways relevant to the tumorigenesis and progression of cancer.

Another important finding was that the G1/S arrest occurred in the HCT116 exposed to the UM1. In response to the MEG3 activation and shRNA against linc-ROR, the apoptosis was induced in the HCT116 cells. These results are consistent with those who suggested that the linc-ROR mediates cell apoptosis through the p53 (34). This lncRNA modulates the p53-regulated processes like cell cycle progression and apoptosis. Hence, it could conceivably be suggested that the induction of apoptosis in the HCT116 cells are attributed to the up-regulation of p53. This study confirms that both lncRNAs are associated with the genes involved in the regulation of cell proliferation. It is possible to hypothesize that the double targeting

genes in one pathway could lead to improve sensitive and intense cell responses in inhibiting cell proliferation, apoptosis and cell cycle arrest.

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no conflicts of interest.

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### Authors' contributions

Prof. Shahbazi initiated and supervised the project. He also helped in the final edition of paper and edited paper. Miss. Ramezani performed all laboratory experiments and analyzed the data. Dr. Shamsabadi involved in the vector design and critically revised this article. All authors approved the final manuscript.

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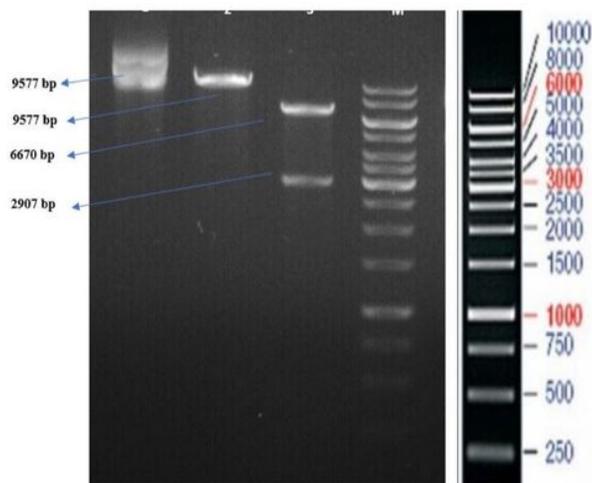
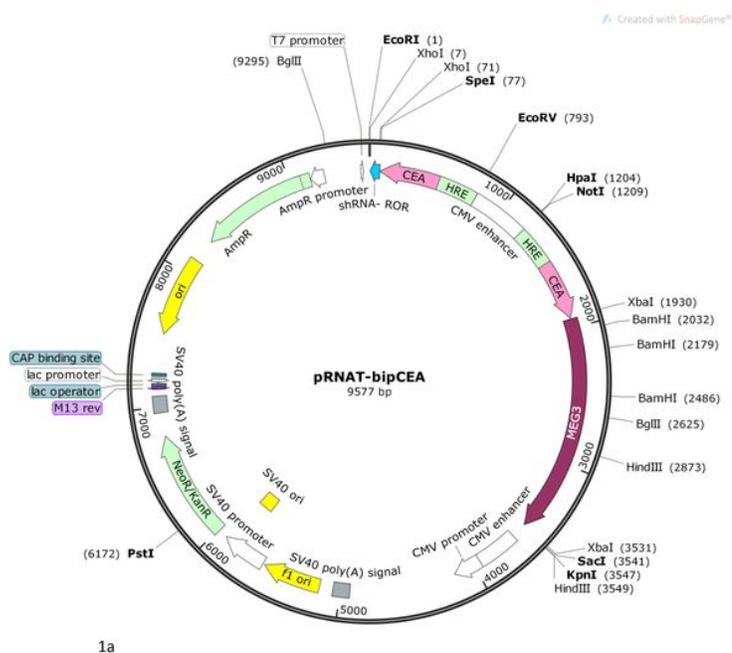
## Table

Table 1.

The characteristics of primers

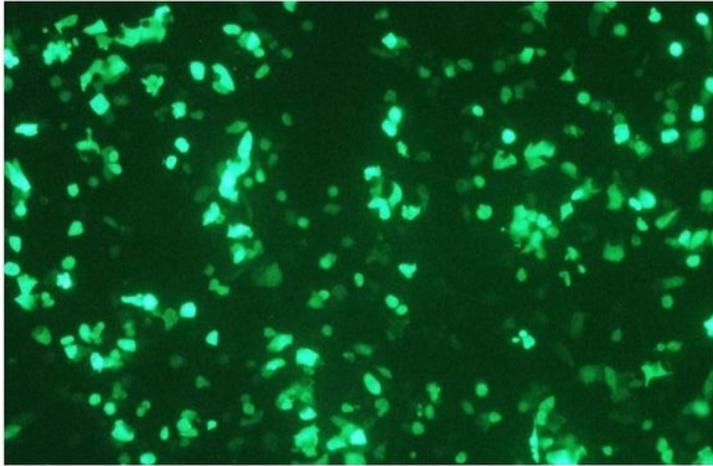
Gene	Primer Sequence(5'-3')	Amplicon Length (bp)
MEG3	F: AGAACTGCGGATGGAAGC	188
NR_00276602	R: TGGCTGTGGAGGGATTTC	
Linc-ROR	F: CAGCAGGTCTCAGGGTTG	174
NR_048536.2	R: AGAGTGGCGATGTGTTTGG	
p53	F: TGAGGTGCGTGTTTGTG	135
NP_000537.2	R: AGAGGAGCTGGTGTGTTGTTG	
ACTB	F: AGCCTCGCCTTTGCCGA	72
NM_001101.5	R: GCGCGGCGATATCATCATC	

## Figures

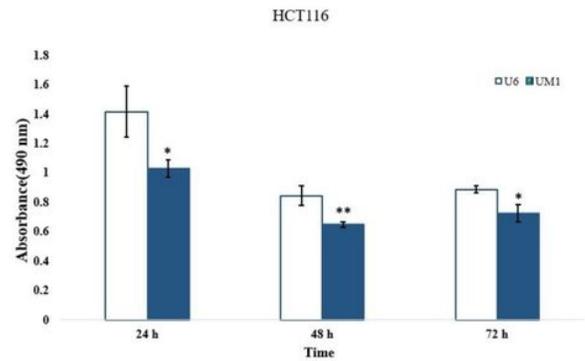


## Figure 1

The map of UM1 vector. (a) The schematic view of the UM1 vector (9577 bp). The MEG3 and linc-ROR expression regulate by the bidirectional CEA promoter. It has been designed by the SnapGene viewer software. (b) The resultant construct was verified by Sac I and ECOR I restriction enzyme digestion.



2a



2b

## Figure 2

UM1 inhibits the proliferation of HCT116 cells. (a) Transfection efficiency was evaluated by the GFP expression. The high expression of GFP was observed at 48h after transfection. (b) A significant reduction of cell viability was observed after 48h post-transfection.

## HCT116

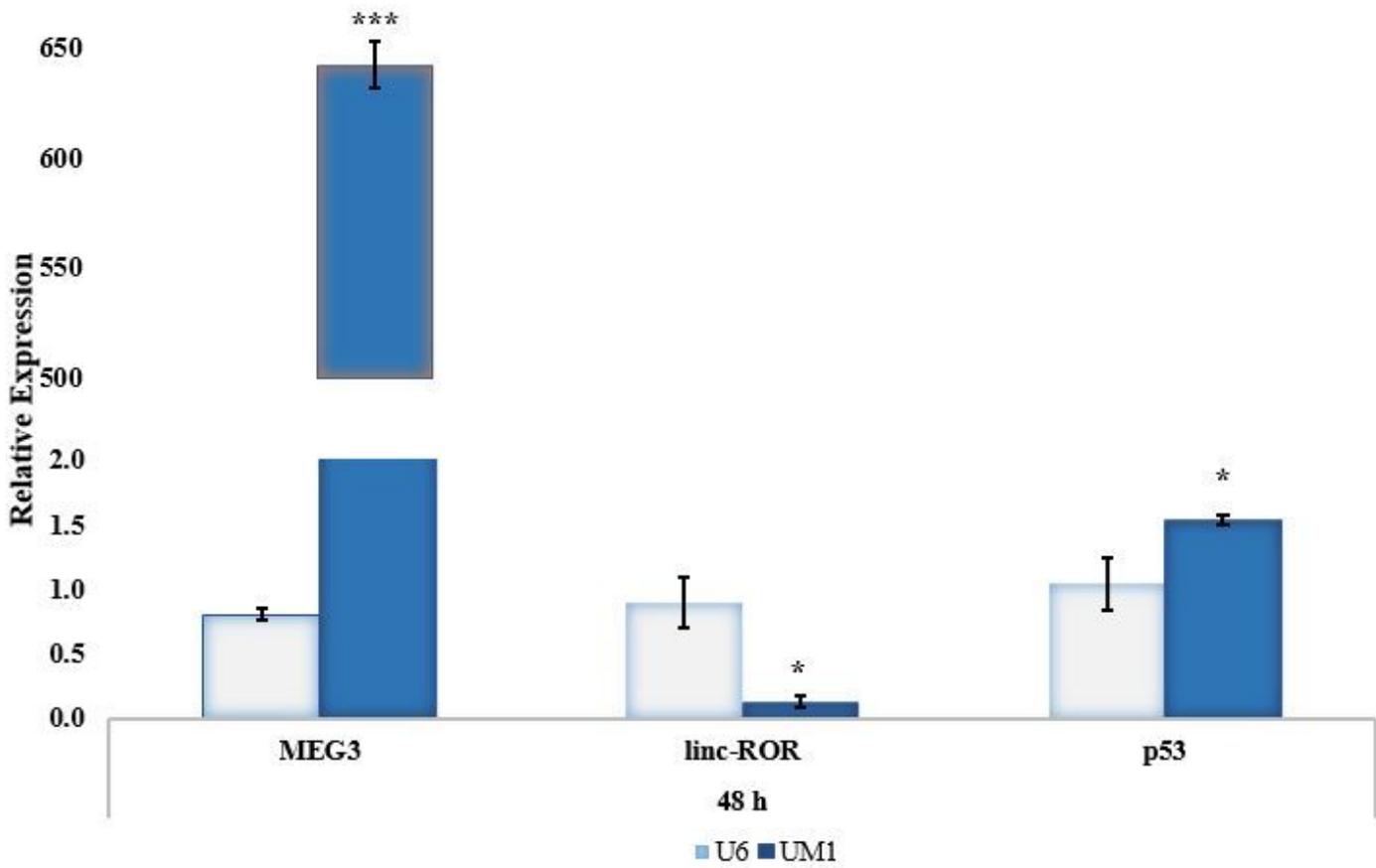
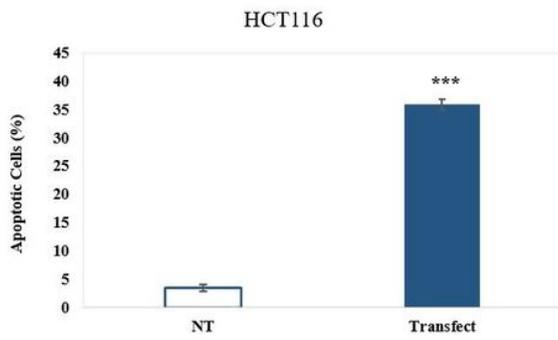
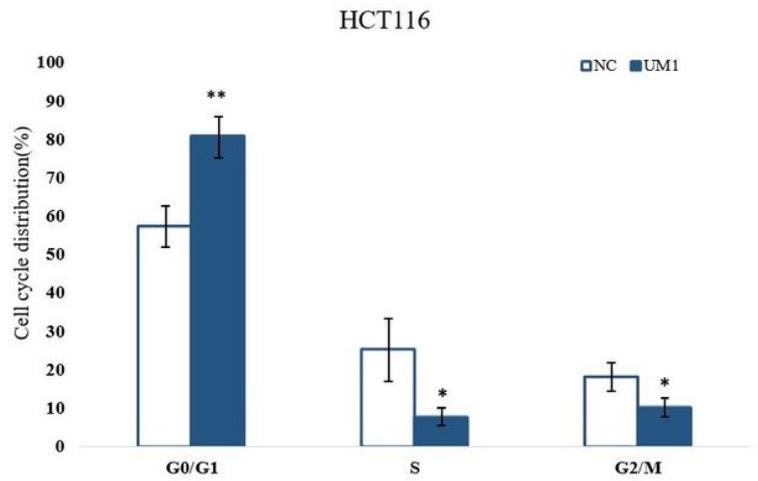


Figure 3

MEG3 and linc-ROR expression in response to UM1 in HCT116 cells. Fold-change expression of the MEG3 was remarkably increased after 48h and 72h post-transfection. Whereas, the expression of linc-ROR was significantly reduced at 48h post-transfection.



4a



4b

**Figure 4**

Apoptosis and cell cycle analysis of HCT116 cells exposed to the UM1. (a) Apoptosis was induced in the transfected HCT116 cell by the simultaneous targeting of MEG3 and linc-ROR (36.35%). (b) The G1/S cell cycle arrest was observed in the HCT116 cells exposed to the UM1. Also, the percentage of cells were significantly decreased in the S phase (7.8) and the G2/M phase (10.7) in the same cells.