Tumor Biology

MicroRNA-138 negatively regulates non-small cell lung cancer cells through the interaction with Cyclin D3

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Full Title:	MicroRNA-138 negatively regulates non-small cell lung cancer cells through the interaction with Cyclin D3
Short Title:	CCND3 regulates NNSCLC
Article Type:	Research Article
Abstract:	Background: Previous studies demonstrate that microRNA-138 (miR-138) is critical in non-small cell lung cancer (NSCLC) regulation. We further explored the molecular mechanism of miR-138 in NSCLC. Methods: Lentivirus was used to upregulate miR-138 in NSCLC cell lines H460 and SPC-A1 cells. Previously known effects of miR-138 upregulation on NSCLC, proliferation, cell cycle division and cisplatin sensitivity, were assessed. Moreover, previously unknown effect of miR-138 upregulation on NSCLC migration was also examined in H460 and SPC-A1 cells. A new miR-138 downstream target, cyclin D3 (CCND3), was assessed by dual-luciferase reporter assay and qRT-PCR. CCND3 was then ectopically over-expressed in H460 and SPC-A1 cells. The effects of forced overexpression of CCND3 on miR-138 induced NSCLC regulations, were further examined by proliferation, cell cycle, cisplatin sensitivity and migration assays, respectively. Results: Lentivirus-induced miR-138 upregulation inhibited NSCLC proliferation and cell cycle division, in line with previous findings. Moreover, we found miR-138 upregulation had other anti-tumor effects, such as increasing cisplatin sensitivity and reducing cancer migration, in H460 and SPC-A1 cells. Luciferase assay and qRT-PCR showed CCND3 was directly targeted by miR-138. Forced overexpression of CCND3 in H460 and SPC-A1 cells reversed the anti-tumor effects of miR-138 upregulation on cancer cell growth, cell cycle, cisplatin sensitivity and migration. Conclusions: Our study revealed novel anti-cancer effects of miR-138 upregulation in NSCLC, as well as its new molecular target of CCND3.

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MicroRNA-138 negatively regulates non-small cell lung cancer cells through the interaction with Cyclin D3

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Abstract

Background: Previous studies demonstrate that microRNA-138 (miR-138) is critical in non-small cell lung cancer (NSCLC) regulation. We further explored the molecular mechanism of miR-138 in NSCLC.

Methods: Lentivirus was used to upregulate miR-138 in NSCLC cell lines H460 and SPC-A1 cells. Previously known effects of miR-138 upregulation on NSCLC, proliferation, cell cycle division and cisplatin sensitivity, were assessed. Moreover, previously unknown effect of miR-138 upregulation on NSCLC migration was also examined in H460 and SPC-A1 cells. A new miR-138 downstream target, cyclin D3 (CCND3), was assessed by dual-luciferase reporter assay and qRT-PCR. CCND3 was then ectopically over-expressed in H460 and SPC-A1 cells. The effects of forced overexpression of CCND3 on miR-138 induced NSCLC regulations, were further examined by proliferation, cell cycle, cisplatin sensitivity and migration assays, respectively.

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Conclusions: Our study revealed novel anti-cancer effects of miR-138 upregulation in NSCLC, as well as its new molecular target of CCND3.

Keywords: NSCLC, miR-138, CCND3, cisplatin

Introduction

Lung cancer is one of the most common cancers in both men and women [1]. In 2014, the estimated newly diagnosed lung cancer patients are above 220,000, and the estimated lung cancer mortality is more than 150,000 in United States [1]. Non-small cell lung cancer (NSCLC) accounts for more than 80% of all lung cancer cases. Once diagnosed, the patients with NSCLC are often found to be in late or advanced stages [2]. Therefore, it is critical to understand the underlying molecular mechanisms of NSCLC, to provide meaningful methods for early diagnosis and optimized treatment strategies for patients with NSCLC.

Recently, studies on epigenetic regulation of microRNA (miRNA) have shown great progress in inhibiting proliferaiton, inducing apoptosis and reducing metastasis in NSCLC [3-7]. Among many of the miRNAs that exert significant regulatory effects in NSCLC, microRNA-138 (miR-138) was shown to be down-regulated in both NSCLC cell lines and NSCLC tumors [3]. Further investigation on functional effects of miR-138 revealed that forced miR-138 upregulation inhibited cancer proliferation, arrested G0/G1 cell cycle and increased cisplatin sensitivity in NSCLC [3, 4, 8]. Interestingly, the down-stream molecular targets of miR-138 was found to be diverse, including polycomb group protein, enhancer of zeste homolog 2 (EZH2) [3], cAMP-dependent kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) [4], and excision repair cross-complementation group 1 protein (ERCC1) [8]. Therefore, it is likely that other genes are involved in miR-138 dependent cancer regulation in NSCLC.

One of the many miR-138 target gene is cyclin D3 (CCND3), which belongs to cell cycle regulator family cyclin D (1-3) and plays important roles in the G-S transition

in cancer cell cycle [9-11]. In colorectal cancer, CCND3 was show to be responsible for the regulation of liver metastasis [12]. In breast cancer and bladder cancer, CCND3 was predicted to be reliable biomarker among cancer patients [13, 14]. In NSCL, CCND3 was shown to be highly associated with cancer risk in patients with NSCLC [15], and reversely regulated by microRNAs m-15a and miR-16 [16, 17]. However, it's still unknown whether CCND3 was associated with miR-138 to exert functional effects on NSCLC regulation.

In the presented work, we constructed lentivirus to up-regulate miR-138 in NSCLC cell lines H460 and SPC-A1 cells. We confirmed the previous observed anti-tumor effects of miR-138 upregulation in NSCLC, including cancer proliferation and cell cycle arrest. Furthermore, we explored new mechanisms of miR-138, including cisplatin sensitivity and migration, in NSCLC regulation. Most importantly, we investigated the molecular association between miR-138 and CCND3 in NSCLC regulation, through dual-luciferase reporter assay, qRT-PCR and ectopic CCND3 overexpression. The results may help further our understanding on the molecular mechanisms of miRNAs in NSCLC.

Materials and methods

NNSCLC cell lines

Two NNSCLC cell lines, H460 and SPC-A1 were purchased from China Academy of Sciences in Shanghai & Shanghai Institute of Cell Biology (Shanghai, China). NNSCLC cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100

U/mL penicillin. All culture reagents were purchased from Invitrogen (Invitrogen, USA). NNSCLC cells were cultured in a humidity-controlled cell incubator at 37 ^{o}C with 5% CO_2, and passaged every 2-3 days.

Lentiviral transfection

Lentivirus containing the oligonucleotides of human mature miR-138 mimics (Len-miR138), and mimic control (Len-C) were purchased from SunBio Biotech (SunBio Medical Biotechnology, Shanghai, China). Lentiviral transfection in H460 and SPC-A1 cells was carried out using Lipofectamine 2000 reagent (Invitrogen, USA) per manufacturer's protocol. Twenty-four hours after transfection, culture medium was replenished and subsequent experiments were carried out.

Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from H460 and SPC-A1 cells using a RNeasy Mini QIAcube Kit on a QIACube (Qiagen, USA) per manufacturer's protocol. After reverse transcription using a TaqMan Reverse Transcription Kit (Qiagen, USA), quantitative real-time PCR (qRT-PCR) was carried out using Taqman Assays and Taqman Universal Master Mix (Qiagen, USA) for miR-138 and CCND3 detection. U6 snRNA and human GAPDH were used as internal controls for miR-138 and CCND3. Gene expression levels were quantified using ($2^{-\Delta\Delta Ct}$) method [18].

Cell proliferation assay

The *in vitro* growth of H460 and SPC-A1 cells was monitored by a Cell Proliferation Reagent Kit I (MTT, Roche Applied Science, USA) per manufacturer's protocol. Briefly, cells were plated in 96-well plate (5000 cells / well). Quantification on cell growth was conducted every day from days 1 to day 5 in the culture, by incubating

the cells with (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h, and washing with 0.1 ml DMSO for 10 mins. The relative optical density (O.D.) was measured at absorbance of 570 nm.

Cell cycle assay

H460 and SPC-A1 cells were plated in 6-well plates. After treatments, cells were collected and fixed by 70% ethanol at RT for 1 h, followed by re-hydration with ice-cold PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide. Cells were then sorted by a FACSort™ cytometer (BD, USA) per manufacturer's protocol. Calculation of cell cycle percentiles was conducted on a MULTICYCLE AV Software (Phoenix Flow System, USA) per manufacturer's protocol.

Cisplatin assay

H460 and SPC-A1 cells were plated in 96-well plates. After treatments, cells were incubated with cisplatin (0, 5, 10, 20, 50 μg/ml) for 12 h, followed by MTT assay to assess the relative survival.

Migration assay

The migration of H460 cells was assessed by a wound-healing assay. Briefly, 24-hour after treatment, cells were re-suspended and re-plated in 6-well plates. Once cells reached ~80% confluency, a plastic 1000 μl Eppendorf pipette tip was used to draw across the centerline of the cultured cells to generate 1 mm wound area. After 24 hours, the migration of H460 cells into the wound area was examined on an Olympus CK2 inverted microscope (Olympus, Japan) with phase contrast objectives.

Dual-luciferase reporter assay

Human cDNA library was used for CCND3 sequencing and amplification through

PCR. The 3'-UTR of wild type (WT) CCND3, including the predicted has-miR-138 binding site, was cloned into a pGL3-basic firefly luciferase plasmid (Promega, USA. A mutated (MU) CCND3 3'-UTR sequence was generated by a QuickchangeXL mutagenesis kit (Strategene, USA) to null the binding of has-miR-138, and then cloned into pGL3-basic plasmid. HEK293T cells were co-transfected with (WT) CCND3 3'-UTR or (MU) CCND3 3'-UTR, and Len-C or Len-miR-138. Twenty-four hours after transfection, the relative firefly luciferase activities were measured by Dual Luciferase Reporter Assay System (Promega, USA) per manufacturer's protocol, and normalized to control with Len-C transfection.

Expression assay

A full-length human CCND3 cDNA was inserted into the EcoRI site of a mammalian expression vector (Invitrogen, USA) with a rapid DNA-ligation kit (Roche Applied Science, USA) to create a CCND3 expression plasmid (pcDNA3.1 /CCND3). The control pCDNA3.1 /+ vector contains no insertion. Then, H460 and SPC-A1 cells were transfected with pcDNA3.1 / CCND3 or pCDNA3.1 /+ with Lipofectamine 2000 reagent (Invitrogen, USA) per manufacturer's protocol. Twenty-four hours after transfection, culture medium was replenished and the following experiments were performed.

Statistical analysis

Each experiment was repeated at least three times. Data were presented as mean $\pm$ S.E.M.. Statistical analysis was performed using SPSS software (Version 18.0, SPSS Inc, USA) with two-tail Student's t-test. Difference was determined as statistically difference if $P < 0.05$.

Results

MiR-138 upregulation inhibits growth and cell division in NSCLC

Previous studies show that miR-138 upregulation was able to inhibit NSCLC tumor growth [3, 4]. In the present study, we firstly constructed lentivirus containing miR-138 mimics (Len-miR138) and transfected two NSCLC cell lines, H460 and SPC-A1 cells with Len-miR138. We also transfected H460 and SPC-A1 cells with a negative control lentivirus Len-C. Twenty-four hours after transfection, we performed qRT-PCR and confirmed that endogenous miR-138 was upregulated in both NSCLC cells (Fig. 1A-B, * $P < 0.05$). We then checked the effect of miR-138 up-regulation on NSCLC growth and cell division. A proliferation assay was performed every day *in vitro* for 5 days and the results showed that cell proliferation was significantly inhibited by miR-138 upregulation in both H460 and SPC-A1 cells (Fig. 1C-D, * $P < 0.05$). A cell division assay also demonstrated that G0/G1 stage was extended in H460 and SPC-A1 cells (Fig. 1E-F). Therefore, our data conformed that miR-138 upregulation inhibits NSCLC proliferation and cell division.

MiR-138 upregulation increases cisplatin sensitivity and decreases migration in NSCLC

We further investigated other functional effects of miR-138 upregulation on NSCLC, including cisplatin sensitivity and migration capability. Firstly, after lentiviral transfection, H460 and SPC-A1 cells were incubated with graded concentration of cisplatin at 0, 5, 10, 20 and 50 μg/ml for 12 h, followed by measurement on cancer survival through a proliferation assay. The results of MTT assay showed that, in both

H460 and SPC-A1 cells, miR-138 upregulation significantly increased cisplatin sensitivities in response to moderate to high concentrations of cisplatin (Fig. 2A-B, * $P < 0.05$). Secondly, we examined the effect of miR-138 upregulation on NSCLC migration capability. We performed a wound-healing assay in H460 cells. The result showed that, 24 hours after wound generation, migration capability of H460 cells into the wound area was significantly inhibited by miR-138 upregulation (Fig. 2C).

CCND3 is regulated by miR-138 in NSCLC

Previous studies showed that PDK1, EZH2 and ERCC1 are the downstream targets of miR-138 in NSCLC regulation [3, 4, 8]. We hypothesized that other genes could also be regulated by miR-138 in NSCLC. We searched online miRNA target prediction software, such as miRNA or TargetScan, and identified that CCND3 is a possible molecular target of miR-138 (Fig. 3A). We performed a dual-luciferase reporter assay and confirmed that CCND3 was targeted by miR-138 (Fig. 3B, * $P < 0.05$). Then we examined whether CCND3 was directly regulated by miR-138 in NSCLC. We transfected H460 and SPC-A1 cells with Len-C or Len-miR138 for 24 hours. The gene expression levels of miR-138 were then assessed by qRT-PCR. The results demonstrated that CCND3 was significantly down-regulated while mi-R138 was up-regulated in H460 and SPC-A1 cells (Fig. 3C-D, * $P < 0.05$).

Overexpressing CCND3 reversed anti-tumor effects by miR-138 upregulation in NSCLC

Since we showed that CCND3 was directly targeted by miR-25, and mediated by miR-138 in NSCLC, we speculated that CCND3 could have functional mechanisms in regulating NSCLC. We transfected H460 and SPC-A1 cells with an expression plasmid

containing CCND3 sequence (pcDNA3.1/CCND3) to ectopically over-express CCND3 in NSCLC cells. In parallel control experiment, H460 and SPC-A1 cells were transfected with an empty expression plasmid (pcDNA3.1/+). Twenty-four hours after transfection, qRT-PCR demonstrated that endogenous CCND3 mRNA was significantly upregulated in both H460 and SPC-A1 cells (Fig. 4A-B, * $P < 0.05$).

We then performed a two-step transfection experiment in NSCLC. H460 and SPC-A1 cells were initially transfected with Len-miR138 to upregulate endogenous miR-138. Twenty-four hours after first transfection, NSCLC cells were transfected for the second time, with either pcDNA3.1/+ or pcDNA3.1/CCND3. The purpose of this experiment was to examine whether CCND3 overexpression (through pcDNA3.1+ transfection) could functionally affect the regulation of miR-138 upregulation (through Len-miR138 transfection). We then followed up the two-step transfection by performing the proliferation assay and cell division assay. The results of proliferation assay demonstrated that CCND3 overexpression restored the cancer cell growth in H460 and SPC-A1 cells (Fig. 4C-D, * $P < 0.05$). The result of cell division assay also showed that CCND3 overexpression released the G0/G1 arrest in H460 and SPC-A1 cells (Fig. 4E-F).

Furthermore, we assessed the effects of over-expressing CCND3 on other miR-138-related regulations in NSCLC. After two-step transfection, a cisplatin sensitivity assay was performed. It showed that CCND3 overexpression significantly restored cisplatin sensitivity in H460 and SPC-A1 cells (Fig. 5A-B, * $P < 0.05$). Finally, we performed a wound-healing assay in H460 cells, and it showed that migration capability was greatly restored by CCND3 overexpression (Fig. 5C).

Overall, our serial two-step transfection experiments all demonstrated that the anti-cancer effects of miR-138 upregulation were all reversed by CCND3 overexpression, thus suggesting that CCND3 was actively involved in miR-138 regulation in NSCLC.

Discussions

MicroRNA-138 has been identified as a critical anti-tumor epigenetic regulator in many cancers, including NSCLC [3, 4, 11, 19-21]. In NSCLC, miR-138 is shown to be down-regulated in both *in vitro* NSCLC cell lines and *in vivo* NSCLC tumors [3]. Subsequent miR-138 upregulation was then shown to have functional roles in NSCLC regulation, such as inhibiting cancer proliferation, inducing G0 cell cycle arrest and increasing cisplatin sensitivity [3, 4, 8]. In our study, we used lentivirus to induce miR-138 ectopic expression in other NSCLC cell lines, H460 and SPC-A1 cells, different than A549 cells used in previous studies [3, 4, 8]. We then observed the miR-138 upregulation inhibited proliferation, extended G0/G1 stages and increased cisplatin sensitivity in H460 and SPC-A1 cells, same as in A549 cells. Thus, our data confirms that the anti-cancer effects of miR-138 are universal across various NSCLC cell lines. Moreover, we performed a wound-healing assay to show that miR-138 upregulation inhibited migration capability in H460 cells. Not only is this the first report to demonstrate that metastasis, a critical characteristic of lung cancer could be affected by miR-138; but also suggests that the anti-tumor effects of miR-138 upregulation in NSCLC could well be predominant as many aspects of cancer development could be altered upon miR-138 upregulation.

A distinctly noticeable aspect of miR-138 regulation in NSCLC is that, the downstream molecular targets are diverse. Studies had revealed various genes to be directly regulated by miR-138 in NSCLC, including including polycomb group protein of enhancer of zeste homolog 2 (EZH2) [3], cAMP-dependent kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) [4], and excision repair cross-complementation group 1 protein (ERCC1) [8]. In our study, we further explored the molecular targets of miR-138 in NSCLC, and discovered through dual-luciferase reporter assay and qRT-PCR that CCND3 was directly targeted by miR-138. Most importantly, we demonstrated, through two-step-transfection experiments, that CCND3 overexpression reversed the anti-tumor effects of miR-138 upregulation, thus exerting oncogenic effect in NSCLC. In a large-scale genome study, CCND3 was identified, among 380 genes, to be highly associated with lung cancer susceptibility [15]. In another study, miR-15a and miR-16 were shown to directly regulate CCDN3 in retinoblastoma –pathway associated manner in NSCLC. Interestingly, aberrant CCND3 regulation on NSCLC has never been reported. And our preliminary data also found little evidence of ectopic CCND3 regulation on cancer development in H460 and SPC-A1 cells (data not shown). Thus, it is very important to note that, in our study, CCND3 overexpression was able to affect NSCLC proliferation, cell cycle, cisplatin sensitivity and migration while miR-138 was up-regulated. It is not clear what the exact mechanisms would be to trigger the regulatory function of CCND3 in NSCLC. Future experiments, such as exploring the molecular mechanism of whole CCND family (CCND1/CCND2/CCND3), may help to reveal the full molecular pathways associated with miR-139 / CCND3 regulation in NSCLC.

In conclusion, our studied identified an important molecular pathway, miR-138 / CCND3, in NSCLC regulation. It may help to explore diagnostic methods and therapeutic reagents for patients with NSCLC.

Conflict of Interest: None.

Figure Legends

Figure 1. The inhibitory effects of miR-138 up-regulation on cancer proliferation and division in NSCLC. H460 and SPC-A1 cells were transfected with miR-138 mimics lentivirus (Len-miR138) or its negative control lentivirus (Len-C). Twenty-four hours after lentiviral transfection, qRT-PCR was performed to examine the gene expression levels of miR-138 in H460 **(A)** and SPC-A1 cells **(B)** (* $P < 0.05$). A proliferation assay was performed for 5 consecutive days to assess the effect of miR-138 upregulation on in vitro cancer growth in H460 **(C)** and SPC-A1 cells **(D)** (* $P < 0.05$). Also after lentiviral transfection, a cell division assay was performed. Relative cell percentiles in G0/G1, S and G2/M stages were calculated for H460 **(E)** and SPC-A1 cells **(F)**.

Figure 2. The effects of miR-138 up-regulation on cisplatin sensitivity and cell division in NSCLC. (A-B) H460 and SPC-A1 cells were transfected with miR-138 mimics lentivirus (Len-miR138) or its negative control lentivirus (Len-C). 24 hours after lentiviral transfection, cells were incubated with 0, 5, 10, 20 and 50 μg/ml cisplatin for 12 h. Relative cell survival was assessed by a proliferation assay in H460 **(A)** and SPC-A1 cells **(B)** (*, $P < 0.05$). **(C)** Also after lentiviral transfection, H460 cells were re-plated in 6-well plates. The migration capability was assessed by a wound-healing assay in 24 h**.**

Figure 3. MiR-138 targeted CCND3 in NSCLC. (A) The diagram of has-miR-138 binding site on wild type (WT) CCND3 3'UTR is shown. A mutation was created at the binding site (MU). **(B)** In a dual-luciferase reporter assay, firefly luciferase reporter

containing WT or MU CCND3 3'UTRs were co-transfected with Len-C or Len-miR138 in HEK293T cells, and the relative luciferase activities were assessed. H460 **(C)** and SPC-A1 **(D)** cells were transfected with Len-C or Len-miR138 for 24 h. The expression levels of CCND3 were assessed by qRT-PCR (*, $P < 0.05$).

Figure 4. The effect of CCND3 upregulation on miR-138 mediated NSCLC growth and cell division. (A-B) H460 and SPC-A1 cells were transfected with a CCND3 expression plasmid (pcDNA3.1/CCND3), or an empty plasmid (pcDNA3.1/+). Twenty-four hours after transfection, CCND3 mRNA levels were examined by qRT-PCR in H460 **(A)** and SPC-A1 **(B)** cells (*, $P < 0.05$). **(C-F)** H460 and SPC-A1 cells were transfected with Len-miR138 for 24 h, then with pcDNA3.1/CCND3 or pcDNA3.1/+ for another 24 h, followed by proliferation assay for H460 **(C)** and SPC-A1 **(D)** cells (*, $P < 0.05$), and cell division assay for H460 **(E)** and SPC-A1 **(F)** cells.

Figure 5. The effect of CCND3 upregulation on miR-138 mediated cisplatin sensitivity and migration in NSCLC. H460 and SPC-A1 cells were transfected with Len-miR138 for 24 h, then with pcDNA3.1/CCND3 or pcDNA3.1/+ for another 24 h. After 2nd transfection, NSCLC cells were treated with 0, 5, 10,20 and 50 μg/ml cisplatin for 12 h, followed by MTT assay to assess cancer cell survival in H460 **(A)** and SPC-A1 **(B)** cells (*, $P < 0.05$). **(C)** H460 cells were re-plated in 6-well plates. The migration capability after two-step transfection was examined by a migration assay**.**

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Figure1
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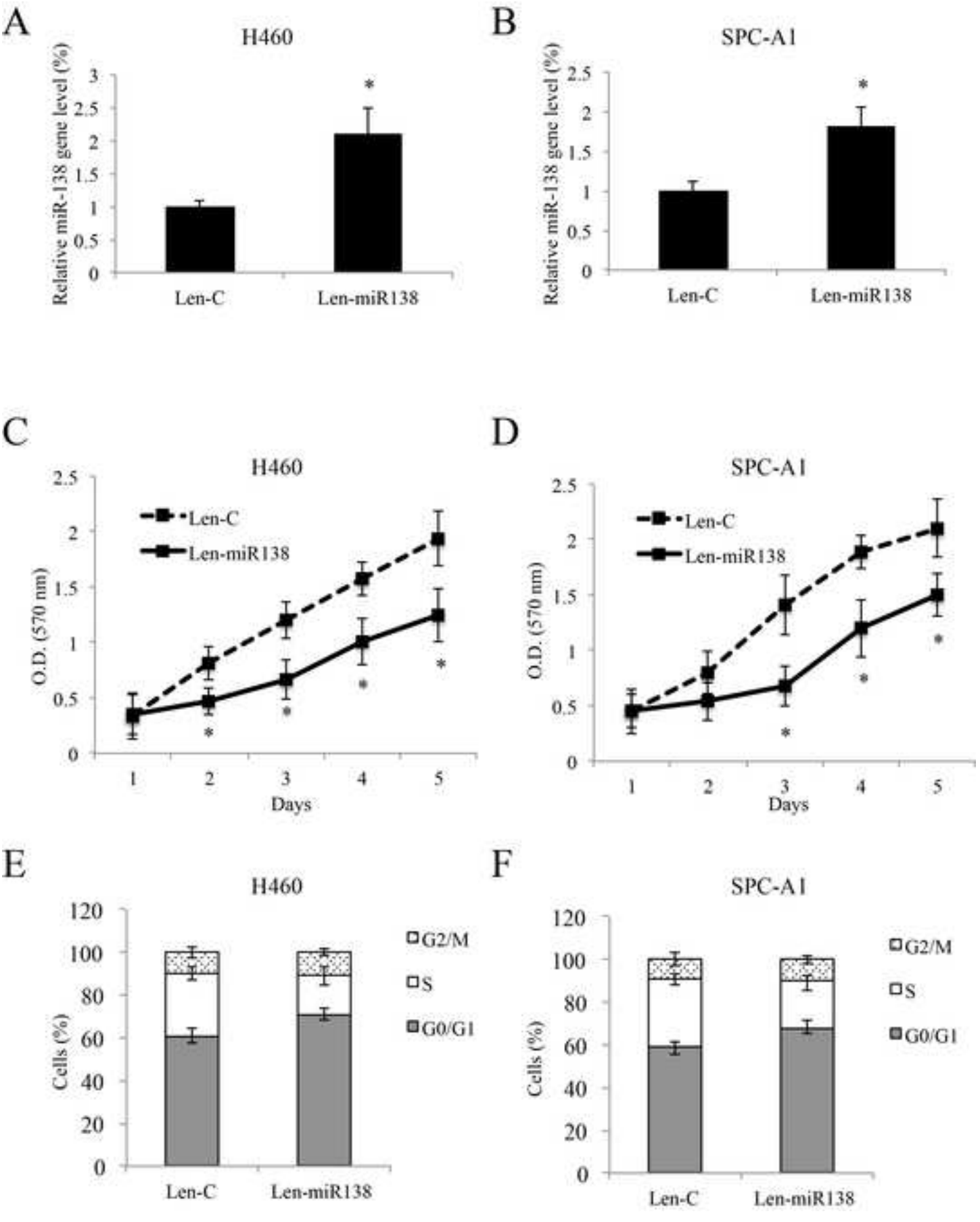

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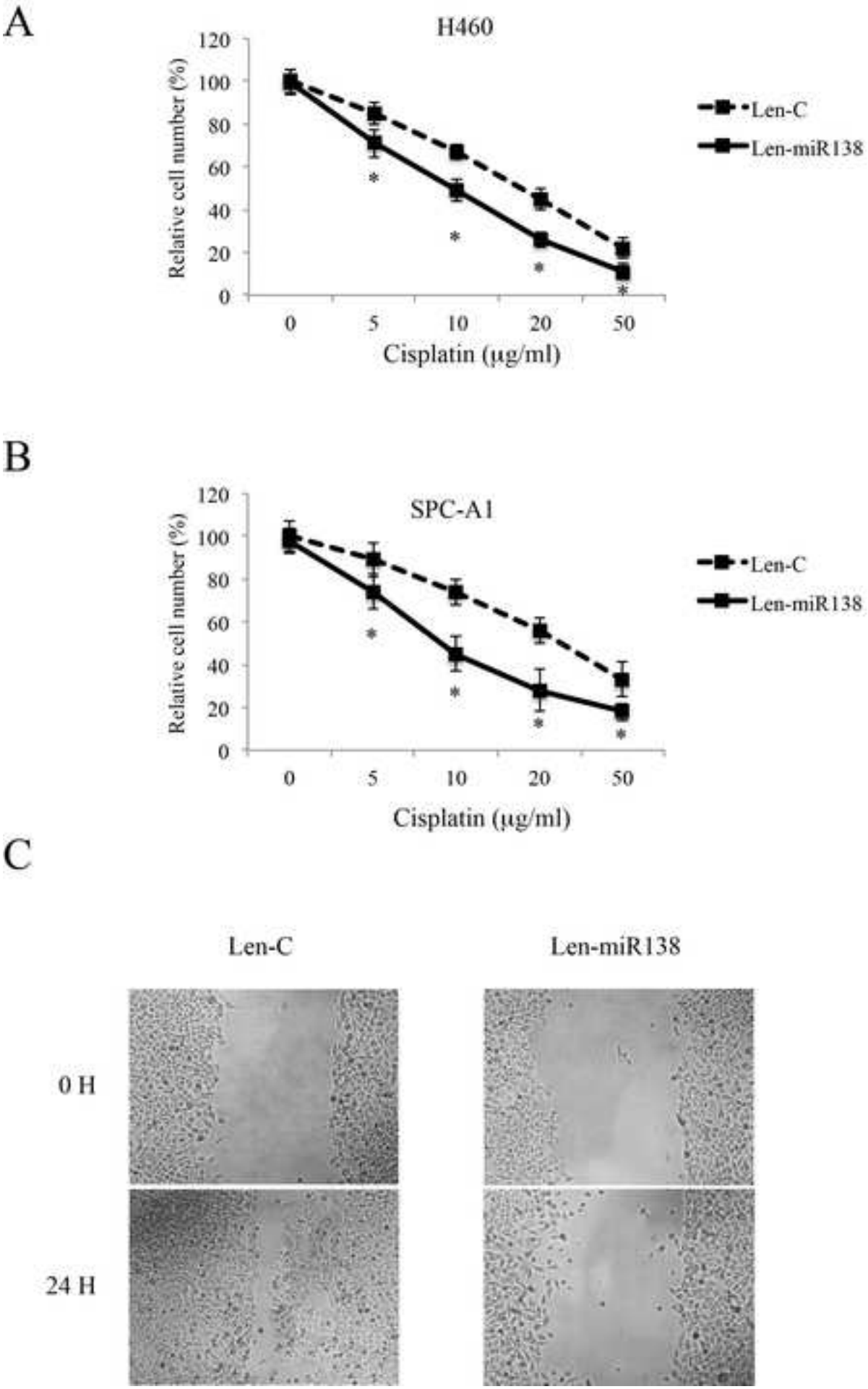

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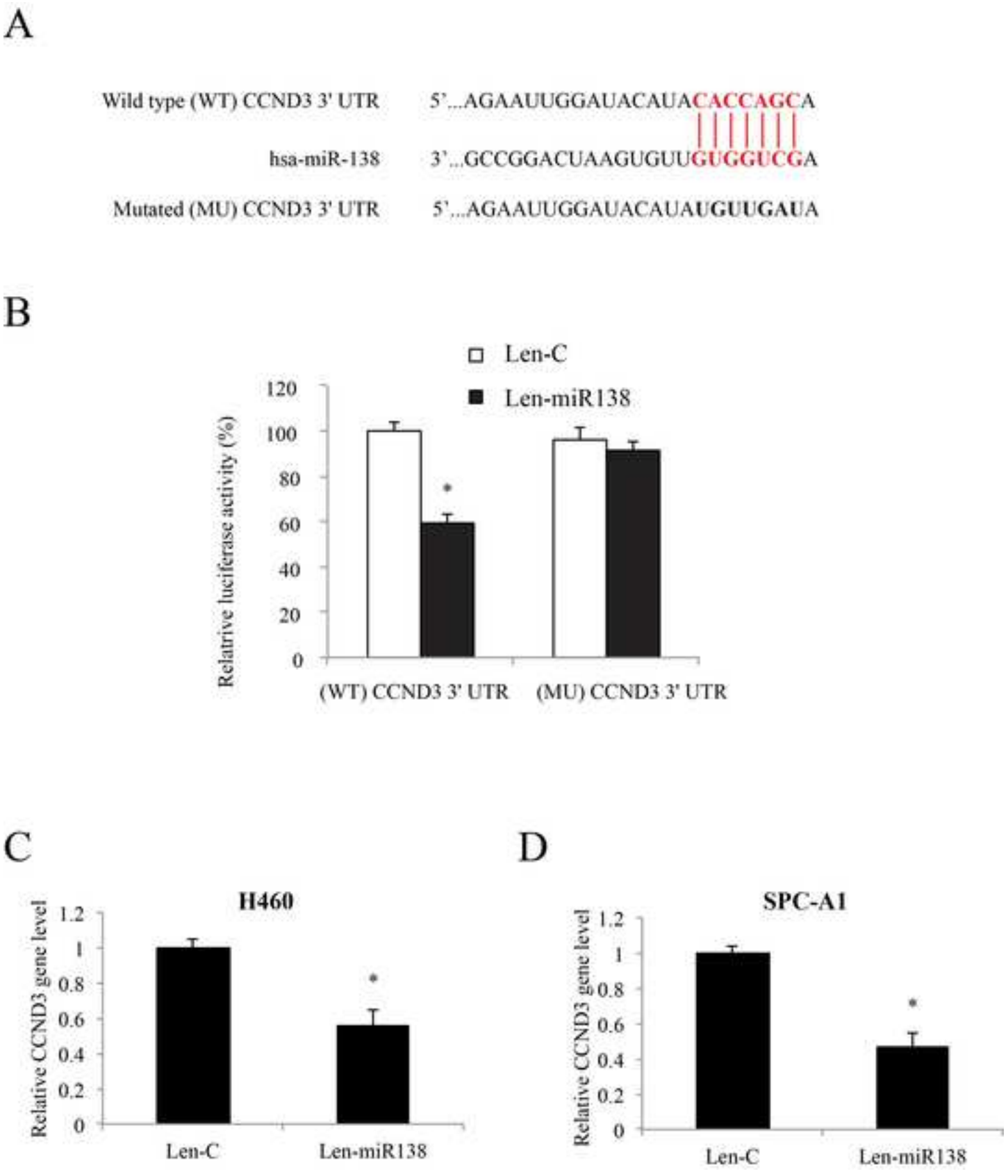

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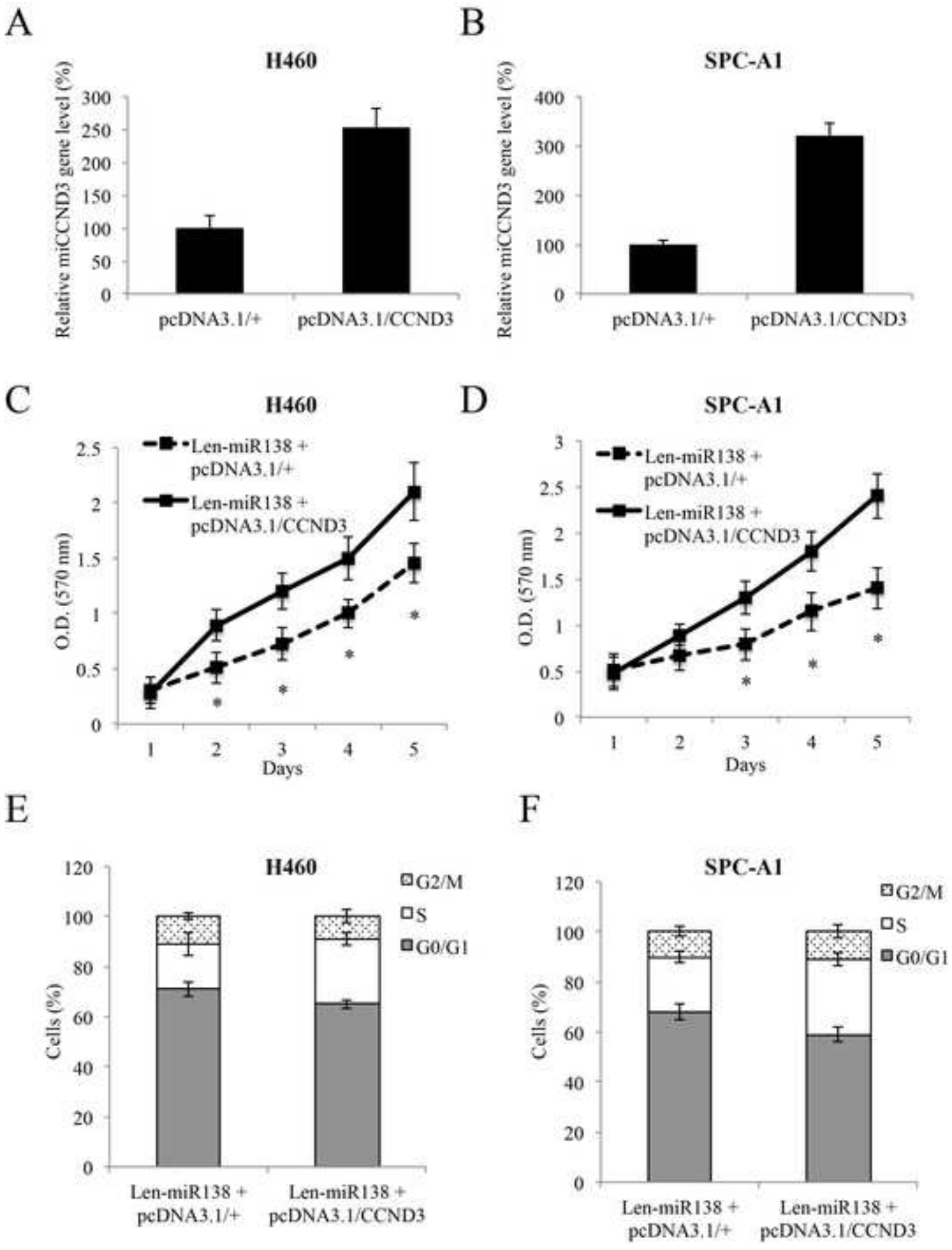

Figure5
Click here to download Figure: Figure05_0318_v2.jpg