Tohoku J. Exp. Med., 2020, **251**, 263-272

MicroRNA Let-7c Contributes to Paclitaxel Resistance via Aurora-B in Endometrial Serous Carcinoma

Izumi Sato,[1,*] Masumi Ishibashi,[1,*] Hideki Tokunaga,[1] Shogo Shigeta,[1] Shoko Sakurada,[1] Muneaki Shimada,[1] Satoru Nagase,[2] Yoh Watanabe[3] and Nobuo Yaegashi[1]

[1]Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[2]Department of Obstetrics and Gynecology, Yamagata University Graduate School of Medical Science, Yamagata, Yamagata, Japan
[3]Department of Obstetrics and Gynecology, Tohoku Medical and Pharmaceutical University, Sendai, Miyagi, Japan

The incidence of endometrial cancer has rapidly risen over recent years. Paclitaxel, a key drug for endometrial cancer treatment, inhibits microtubule depolymerization and induces apoptosis in cancer cells. Endometrial serous carcinoma (ESC) accounts for < 10% of all endometrial carcinomas, but its aggressive nature makes it responsible for close to 40% of cancer deaths. Thus, novel therapeutic targets are required for ESC. To identify microRNAs that promote paclitaxel resistance, we established two paclitaxel-resistant cell lines from USPC1 human ESC cells by exposing paclitaxel to parental cells for 12 weeks. Paclitaxel concentrations were increased every 2 weeks, and after 12 weeks of paclitaxel exposure, two replicate paclitaxel-resistant cell lines were established (USPC1-PTSR1 and USPC1-PTXR2). The microarray analysis was performed using USPC1 cells and USPC1-PTXR1 cells, and eight candidate microRNAs were thus selected as potential mediators of paclitaxel sensitivity. Among these candidate microRNAs, let-7c precursor treatment of paclitaxel-resistant USPC1-PTXR1 cells caused the greatest increase in paclitaxel-mediated cytotoxicity. Let-7c inhibition conversely decreased paclitaxel-induced apoptosis. It is known that let-7a microRNA, a member of the let-7 family, inhibits growth of endometrial carcinoma cells targeting Aurora-B that controls progression through each phase of mitosis. We thus studied whether let-7c mediates Aurora-B expression in ESC cells. The expression levels of Aurora-B mRNA and protein were higher in USPC-PTXR1 cells compared with USPC1 cells. Let-7c inhibition increased Aurora-B expression in USPC1 cells but decreased Aurora-B expression in USPC1-PTXR1 cells. These results indicate that let-7c mediates paclitaxel resistance via inhibition of Aurora-B expression in ESC cells.

Keywords: Aurora-B; endometrial serous carcinoma; let-7c; microRNA; paclitaxel
Tohoku J. Exp. Med., 2020 August, **251** (4), 263-272.

Introduction

The incidence of endometrial cancer in the United States has risen rapidly over recent years. In 2019, there were estimated 61,880 cases and 12,160 deaths, making uterine cancer the fifth most common cause of female cancer death in the United States (Siegel et al. 2019). Endometrial serous carcinoma (ESC) accounts for < 10% of all endometrial carcinomas. However, its aggressive nature makes it responsible for close to 40% of cancer deaths (Fader et al. 2010). ESC is commonly treated by comprehensive surgical staging followed by carboplatin

Received February 19, 2020; revised and accepted July 3, 2020. Published online July 30, 2020; doi: 10.1620/tjem.251.263.
*These two authors contributed equally to this work.
Correspondence: Masumi Ishibashi, M.D., Ph.D., Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan.
e-mail: masumi.ishibashi.d6@tohoku.ac.jp
Hideki Tokunaga, M.D., Ph.D., Department of Obstetrics and Gynecology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan.
e-mail: tokunagahideki@med.tohoku.ac.jp

and paclitaxel chemotherapy. Even so, ESC demonstrates higher rates of recurrence and a tendency for distant, extrauterine metastases compared with other endometrial cancer subtypes (Chang-Halpenny et al. 2013). Thus, novel therapeutic targets for chemotherapy-resistant ESC are needed.

MicroRNAs are small noncoding RNAs (approximately 18-24 nucleotides) that negatively regulate gene expression post-transcriptionally by interfering with the translation of one or more target mRNAs (Pritchard et al. 2012). MicroRNA dysregulation contributes to cancer pathogenesis. Various microRNA replacement therapies are currently in clinical trials, which demonstrate the potential of this approach to treat cancer (Hosseinahli et al. 2018).

Lethal-7 (let-7) was the second discovered microRNAs family in *Caenorhabditis elegans* and the first discovered microRNAs subsequently in human. In human, this family encompasses 13 member (let-7a-1, 7a-2, 7a-3, 7b, 7c, 7d, 7e, f7-1, 7f-2, 7g, 7i, mir-98, and mir-202) (Ruby et al. 2006). Recent studies have reported that let-7 family members generally promote differentiation during development and function as tumor suppressors in various cancers (Reinhart et al. 2000; Takamizawa et al. 2004; Grosshans et al. 2005; Johnson et al. 2005; Kumar et al. 2007). Let-7c has been reported as a tumorigenesis suppressor and that enhances the chemosensitivity of human mucosal melanoma (Tang et al. 2019). It has also been reported that let-7c is involved in prostate cancer pathogenesis (Mulholland et al. 2019).

Aurora-B is a serine/threonine kinase that controls progression through each phase of mitosis. Its primary roles are in the construction of mitotic spindle, segregation of chromosomes, and completion of cytokinesis (Fu et al. 2007). It has been reported that let-7a inhibits the endometrial carcinoma by targeting Aurora-B (Liu et al. 2013), and Aurora-B activity is known to modulate taxane response in non-small cell lung cancer (Al-Khafaji et al. 2017).

Let-7c and Aurora-B is known to contribute to chemosensitivity respectively. However, it has not been clear whether let-7c and Aurora-B cooperatively mediate paclitaxel resistance in ESC or not. Based on the evidence that another let7 family member let-7a targets Aurora-B, we hypothesized that let-7c might contribute to paclitaxel resistance by inhibiting Aurora B expression in ESC cells.

In the current study, we established paclitaxel-resistant ESC cell lines and investigated the mechanisms by which let-7c microRNA contributes to paclitaxel resistance focusing on the interaction with aurora B.

Materials and Methods

Reagents

Aurora-B-specific antibody (A00780) was purchased from Genscript (Piscataway, NJ, USA). Antibodies specific for poly ADP-ribose polymerase (PARP) (sc7150) and cleaved-caspase-3 (9661) were purchased from Cell Signaling Technology (Boston, MA, USA). β-actin antibody (A5441) was purchased from Sigma (St. Louis, MO, USA). Paclitaxel and cisplatin were purchased from Wako (Japan).

The human ESC cell line USPC1 was provided by Dr. A. Santin at the Department of Obstetrics and Gynecology, Division of Gynecologic Oncology at the Yale University School of Medicine.

MicroRNA inhibitors specific for miR-1290, miR-200b, miR-1246, and miR-1268a were purchased from Sigma-Aldrich (St. Louis, MO, USA). MicroRNA precursors specific for miR-125b, miR-99a, let-7c, and let-7b-5p were purchased from Ambion (Austin, TX, USA).

Cell culture

The ESC cell lines (USPC1, USPC1-PTXR, USPC1-PTXR2) were cultured in Roswell Park Memorial Institute 1640 medium (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and antibiotics and antimycotics at the following dilution: 1% penicillin, streptomycin, amphotericin B, and GlutaMAX (Gibco/Life Technologies).

Establishing a paclitaxel-resistant cell line

To establish paclitaxel-resistant clones from the USPC1 cell line, paclitaxel was added to the culture medium, beginning with 5 nM for 2 w. The paclitaxel concentration was increased every 2 w over 10 w at the following concentrations: 10, 50, 100, 200, and 400 nM. Cultures received a total of 12 w of paclitaxel exposure to establish two replicate lines (USPC-PTXR1 and USPC1-PTXR2) (Fig. 1A). Parental USPC1 cells were cultured at 37°C, 5% CO2 over 12 w without adding paclitaxel. Cell viability was assessed using the Cell Counting Kit-8 (Dojindo, Japan) according to the manufacturer's instructions.

MicroRNA microarray analysis

Total RNA was extracted from cells using a miRNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentration was determined by measuring absorbance using a NanoDrop ND1000 instrument (Thermo Fisher Scientific, Loughborough, UK) and confirmed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). MicroRNA microarray analysis, including labeling, hybridization, scanning, normalization, and data analysis, was carried out according to the manufacturer's instructions. The arrays were scanned with an Agilent Microarray Scanner (Agilent Technologies). Images were extracted using Feature Extraction software 10.10.1.1 and analyzed by Agilent GeneSpring GX 12.6.0 (Agilent Technologies). According to our previously published protocol, microRNAs that had a greater than 1.5-fold change (fold change > 1.5 or < 0.67) were considered to have significant differential expression compared with parental cells.

MicroRNA precursor and inhibitor transfection

Cells were transfected with a microRNA inhibitor (for

miR-1290, miR-200b, miR-1246, and miR-1268a), microRNA precursor (for miR-125b, miR-99a, let-7c, and let-7b-5p), or their respective negative controls using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's instructions.

Quantitative real-time PCR

Cell homogenization and RNA extraction were performed using an ISOGEN II kit (Nippongene, Japan) according to the manufacturer's instructions. Extracted RNAs (1 μg) were reverse transcribed to generate cDNAs using SuperScript III (Thermo Fisher Scientific). Real-time PCR was performed using the StepOnePlus Real-Time PCR System with TaqMan Universal Master Mix II and a TaqMan probe specific for each gene (Thermo Fisher Scientific) to determine let-7c, Aurora-B, and GAPDH mRNA expression.

Western blotting

Cells were washed with ice-cold PBS three times. Harvested cells were lysed using Laemmli buffer. Cellular proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 5%-20% gradient gel. Proteins were electrophoretically transferred to nitrocellulose membranes then blocked with 0.1% Tween 20 in PBS (PBS-T) containing 5% nonfat dried milk. The membranes were incubated with primary antibodies at 4 °C overnight. The blots were washed with PBS-T and incubated with a horseradish peroxidase-conjugated secondary antibody in PBS-T containing 5% nonfat dried milk. The proteins were detected using SuperSignal West Dura Extended Duration Substrate and ChemiDoc MP (Bio-Rad).

Immunofluorescence

Indirect immunofluorescence microscopy was performed following a modified method (Kitatani et al. 2016:Ishibashi et al. 2018). Briefly, cells were plated on glass-bottomed 35 mm dishes and washed with PBS, fixed with 4% formaldehyde in PBS for 10 min at room temperature, then treated with 0.1% Triton-X100 for 10 min. Cells were blocked with 20% human serum in PBS for 1 h followed by primary antibody treatment in PBS containing 20% human serum at 4 °C overnight. The cells were washed with PBS, treated with Alexa488- and/or Alexa555-conjugated anti-IgG antibodies in PBS containing 20% human serum for 1 h, then stained with Hoechst 33342. Confocal laser microscopy was performed using an LSM780 confocal microscope (Carl Zeiss, Thornwood, NY, USA).

Statistical analysis

Values are presented as the mean ± standard error of the mean based on at least three independent experiments. Images are representative of at least three independent experiments. Statistical analyses were performed using GraphPad Prism 6(GraphPad Software, San Diego, CA, USA). Statistical significance was assessed using individual t-tests with $P < 0.05$ considered significant.

Results

Microarray analysis identifies candidate microRNAs that mediate paclitaxel sensitivity in ESC cells

To identify microRNAs that promoted paclitaxel resistance, two replicate paclitaxel-resistant cell lines (USPC1-PTXR1 and USPC1-PTXR2) were established from USPC1 cells. The half maximal inhibitory concentration (IC50) for USPC1, USPC1-PTXR1, and USPC1-PTXR2 was 5.15, 504.48, and 583.59 nM, respectively (Fig. 1B). The cell growth of USPC1-PTXR1 and USPC1-PTXR2 cells were significantly degraded than the USPC1 cells (Fig. 1C).

Microarray analysis was performed using USPC1 and USPC1-PTXR1 cells. Seventeen microRNAs were up-regulated and 45 microRNAs were down-regulated in USPC1-PTXR1 cells compared with USPC1 cells (Fig. 1D, E). The largest up-regulated fold changes were observed in miR-1290, miR-200b-3p, miR-1246 and miR-1268a with 9.74, 4.40, 3.71 and 3.60 fold changes, respectively. The largest down-regulated fold changes were observed in miR-125b-5p, miR-99a-5p, let-7c and let-7b-5p with 14.3, 9.90, 7.26 and 4.38, respectively. Therefore, these were determined as candidate microRNAs.

Let-7c as a candidate therapeutic target for paclitaxel-resistant ESC cells

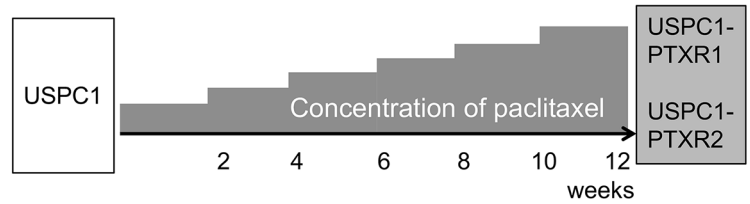
To validate the eight therapeutic target candidate microRNAs, we performed inhibitor treatment for the up-regulated microRNAs and precursor treatment for the down-regulated microRNAs in USPC1-PTXR1 cells. Inhibitor treatment did not produce significant IC50 changes in the up-regulated microRNAs. However, of the down-regulated microRNAs, let-7c precursor treatment produced the greatest increase in paclitaxel-mediated cytotoxicity (Fig. 2B). The let-7c precursor treatment effect was replicated in USPC1-PTXR2 cells (Fig. 2C).

To confirm that let-7c is involved in paclitaxel resistance in ESC, USPC1 cells were treated with paclitaxel and the let-7c inhibitor and the IC50 was calculated. The IC50 was 15.0 nM in negative control USPC1 cells and 25.5 nM in let-7c inhibitor-treated cells (Fig. 2D), indicating that let-7c inhibitor treatment desensitized cells to paclitaxel. These results suggest that let-7c might be a therapeutic target in paclitaxel-resistant ESC.

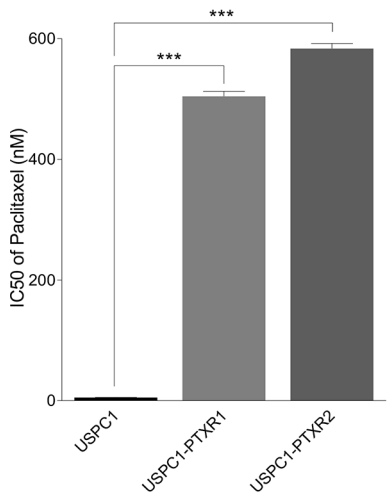
Let-7c inhibition decreases paclitaxel-induced apoptosis

Paclitaxel inhibits microtubule depolymerization, selectively arrests cells in the G2/M phase of the cell cycle, and induces apoptosis in cancer cells (Barbuti and Chen 2015). Let-7c inhibition significantly inhibited paclitaxel-induced PARP cleavage (Fig. 3A, B), and tended to inhibit paclitaxel-induced caspase-3 cleavage (Fig. 3A, C). These results indicate that let-7c inhibition provides an inhibitory effect on paclitaxel-induced apoptosis.

A

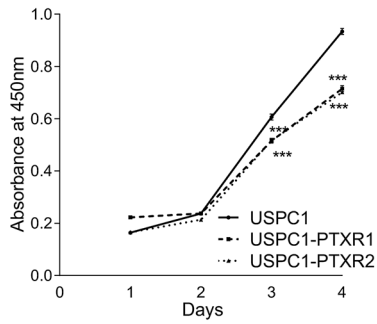


B



	USPC1	USPC1-PTXR1	USPC1-PTXR2
IC50 (nM)	5.15	504.48	583.59

C



D

Up regulated microRNAs

name	Fold change
hsa-miR-1290	**9.74476**
hsa-miR-200b-3p	**4.40136**
hsa-miR-1246	**3.71576**
hsa-miR-1268a	**3.60173**
hsa-miR-31-5p	2.71514
hsa-miR-29b-1-5p	2.36520
hsa-miR-183-5p	2.02698
hsa-miR-31-3p	1.85890
hsa-miR-29a-3p	1.84133
hsa-miR-320c	1.80159
hsa-miR-320d	1.71681
hsa-miR-320e	1.70164
hsa-miR-320b	1.68879
hsa-miR-3135b	1.67277
hsa-miR-25-3p	1.64983
hsa-miR-320a	1.63986
hsa-miR-93-5p	1.51703

E

Down regulated microRNAs

name	Fold change
hsa-miR-125b-5p	**14.34321**
hsa-miR-99a-5p	**9.90100**
hsa-let-7c	**7.26791**
hsa-let-7b-5p	**4.38299**
hsa-miR-374a-5p	4.11777
hsa-miR-19a-3p	2.84276
hsa-miR-15a-5p	2.72820
hsa-let-7d-5p	2.67675
hsa-miR-26a-5p	2.65367
hsa-miR-494	2.60543
hsa-miR-130b-3p	2.48635
hsa-miR-130a-3p	2.39405
hsa-let-7a-5p	2.33081
hsa-miR-10a-5p	2.32574
hsa-miR-19b-3p	2.30368
hsa-miR-16-5p	2.21580
hsa-miR-135b-5p	2.13478
hsa-miR-30d-5p	2.12989
hsa-miR-98-5p	2.07886
hsa-miR-939-5p	2.01515
hsa-let-7f-5p	1.99631
hsa-miR-15b-5p	1.96834
hsa-miR-18a-5p	1.96183
hsa-miR-374b-5p	1.93919
hsa-miR-27b-3p	1.93672
hsa-miR-18b-5p	1.85473
hsa-miR-210	1.74625
hsa-miR-23b-3p	1.73348
hsa-miR-23a-3p	1.70501
hsa-miR-1280_v18.0	1.67976
hsa-miR-4672	1.67779
hsa-miR-17-5p	1.67746
hsa-miR-196b-5p	1.67501
hsa-miR-20a-5p	1.67290
hsa-miR-20b-5p	1.65665
hsa-miR-30b-5p	1.65316
hsa-miR-128	1.64496
hsa-miR-574-5p	1.62998
hsa-miR-5100	1.62934
hsa-miR-193a-3p	1.62667
hsa-let-7g-5p	1.58624
hsa-miR-1260a	1.55399
hsa-miR-361-5p	1.52446
hsa-let-7i-5p	1.51879
hsa-miR-21-3p	1.51035

Fig. 1. Microarray-identified microRNAs that mediate paclitaxel sensitivity in endometrial serous carcinoma cells.
A. The schematic schedule for establishing paclitaxel-resistant cells.
B. IC50 for USPC1, USPC1-PTXR1, and USPC-PTXR2 cell lines. Cells were plated in 96-well plates and treated with paclitaxel for 72 h. The results were expressed as the means ± SEM values from three assays. The values of absorbance of each cells were compared using unpaired t-test.
***P< 0.005
C. The proliferation rates of the USPC1-PTXR1 and USPC1-PTXR2 cells were similar to that of the USPC1 cells. USPC1, USPC1-PTXR1 and USPC1-PTXR2 cells were plated in 96-well plates and cell growth were determined using Cell Counting Kit-8. The results were expressed as the means ± SEM values from three assays. The values of absorbance of each cells were compared using unpaired t-test.
***P< 0.005
D. Microarray analysis was performed in USPC1 and USPC1-PTXR1 cells. Seventeen microRNAs were significantly up-regulated in USPC1-PTXR1 cells compared with USPC1 cells. The microRNAs with the four largest up-regulated fold changes were selected as candidates.
E. Forty-five microRNAs were down-regulated in USPC1-PTXR1 cells compared with USPC1 cells. The microRNAs with the four largest down-regulated fold changes were selected as candidates.

It is also known that paclitaxel-induced microtubule depolymerization disrupts mitotic spindle formation, resulting in the failure of cells to complete normal mitosis (Jordan et al. 1991). Polynuclear formation is one result of mitosis failure in response to paclitaxel-induced microtubule depolymerization (Gascoigne and Taylor 2009). Let-7c inhibition decreased paclitaxel-induced polynuclear formation (Fig. 3D). These results suggest that let-7c inhibition may reduce apoptosis by inhibiting paclitaxel-induced microtubule depolymerization.

Let-7c might contribute to paclitaxel resistance by inhibition of Aurora-B expression in ESC cells

Aurora-B activity is known to modulate taxane response in non-small cell lung cancer(Al-Khafaji et al. 2017), and it is also known that let-7a microRNA inhibits cell growth of endometrial carcinoma targeting Aurora-B(Liu et al. 2013). Based on these previous findings, we studied whether let-7c mediates Aurora-B expression in ESC cells.

To define the molecular mechanisms underlying let-7c-mediated paclitaxel sensitivity, we compared Aurora-B expression levels in USPC1 and USPC1-PTXR1 cells. Aurora-B mRNA and protein expression was higher in USPC-PTXR1 cells compared with USPC1 cells (Fig. 4A, B). Let-7c inhibition increased Aurora-B expression in USPC1 cells (Fig. 4C, D) but decreased Aurora-B expression in USPC1-PTXR1 cells (Fig. 4E, F). Overall, these results suggest that let-7c mediates paclitaxel resistance via inhibition of Aurora-B expression in ESC cells.

Discussion

There is an urgent need to discover novel therapeutic strategies for chemo-resistant cancer. MicroRNAs regulate drug resistance in various cancers (Si et al. 2019). The current study demonstrated that inhibition of the microRNA let-7c desensitized ESC cells to paclitaxel and up-regulated Aurora-B expression. Therefore, let-7c may represent a novel therapeutic target for patients with paclitaxel-resistant ESC.

Let-7c has been reported to inhibit cell proliferation in human hepatocellular carcinoma (Zhu et al. 2015) and cholangiocarcinoma (Xie et al. 2018). Our study is the first to present a relationship between let-7c and paclitaxel resistance in ESC.

The cellular target for paclitaxel has been identified as β-subunit of tubulin in microtubules. Mutations in β-tubulin can disrupt microtubule assembly, conferring taxane resistance (Yin et al. 2010). It has been reported that restoration of miR-200c enhanced sensitivity to paclitaxel in endometrial, breast and ovarian cancer cell lines, and one of the direct target of miR-200 is β-tubulin (Cochrane et al. 2009). In this study, miR-200b was up-regulated in paclitaxel-resistant ESC cells. It seems that miR-200 is one of the therapeutic target for paclitaxel-resistant ESC, but let-7c showed higher anti-tumor effect for paclitaxel-resistant ESC cells than miR-200 in our study.

Previous studies have indicated a link between the let7 family, Aurora-B, and cancer. Aurora-B is a serine/threonine kinase that controls progression through each phase of mitosis. Its primary roles are in the construction of mitotic spindle, segregation of chromosomes, and completion of cytokinesis (Fu et al. 2007). Aurora-B ensures the proper attachment of chromosomes to the microtubules of the mitotic spindle by correcting microtubules attached to the wrong kinetochore (merotelic attachments). The inability to correct these mis-attachments is a major source of chromosome instability in cancer cells, which is an underlying cause of aneuploidy (Noujaim et al. 2014). Aurora-B activity is known to modulate taxane response in non-small cell lung cancer cells (Al-Khafaji et al. 2017), let7 microRNAs repress Aurora-B and regulate cell proliferation (Johnson et al. 2007), and let-7i inhibits malignant cell phenotypes in part by targeting Aurora-B in osteosarcoma cells (Zhang et al. 2015). Based on the previous findings, we have further examined whether let-7c regulates Aurora-B expression. The current study indicated that let-7c negatively regulates Aurora-B expression. However, further studies like target prediction and reporter assays are necessary to confirm that Aurora-B is a direct target of let-7c.

ESC demonstrates higher rates of recurrence and a tendency for distant, extrauterine metastases compared with

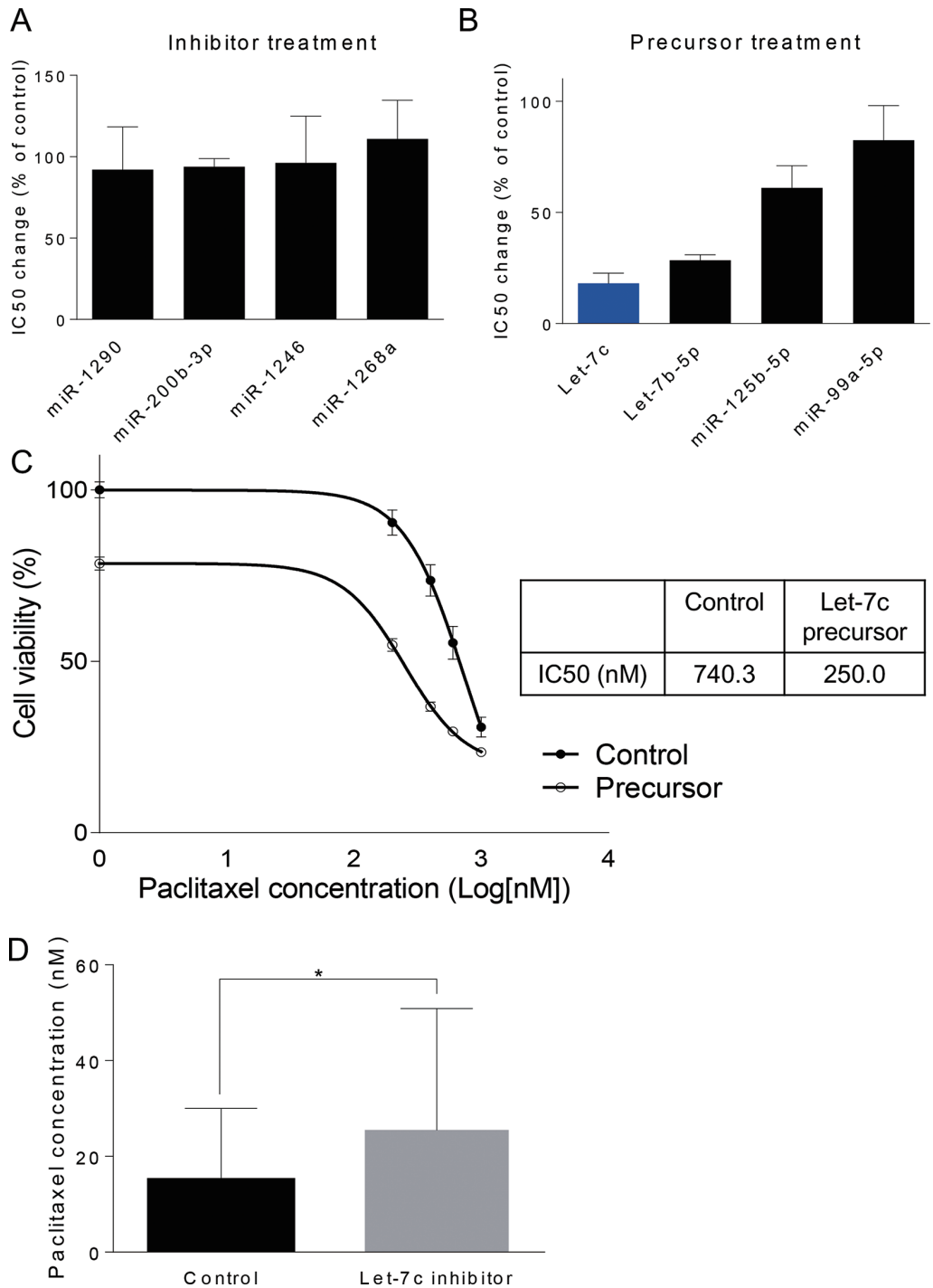


Fig. 2. Let-7c as a therapeutic target for paclitaxel-resistant endometrial serous carcinoma cells.

A. USPC-PTXR1 cells were treated with inhibitors that targeted the up-regulated microRNAs. USPC1-PTXR1 cells were plated in a 96-well plate and transfected with each inhibitor for 24 h before being treated with paclitaxel for 72 h. Cell viability was assessed using the Cell Counting Kit-8 and IC50 values were determined.

B. USPC-PTXR1 cells were treated with precursors that targeted the down-regulated microRNAs. USPC1-PTXR1 cells were plated in a 96-well plate and transfected with each precursor for 24 h before being treated with paclitaxel for 72 h. Cell viability was assessed using the Cell Counting Kit-8 and IC50 values were determined.

C. USPC1-PTXR2 cells were plated in a 96-well plate and transfected with let-7c precursor and control precursor for 24 h before being treated with paclitaxel for 72 h. Cell viability was assessed using the Cell Counting Kit-8 and IC50 values were determined.

D. USPC1 cells were plated in a 96-well plate and transfected with let-7c inhibitor and control inhibitor for 24 h before being treated with paclitaxel for 72 h. Cell viability was assessed using the Cell Counting Kit-8 and IC50 values were determined. IC50 values represent the mean ± SEM of three independent experiments.

*$P < 0.05$

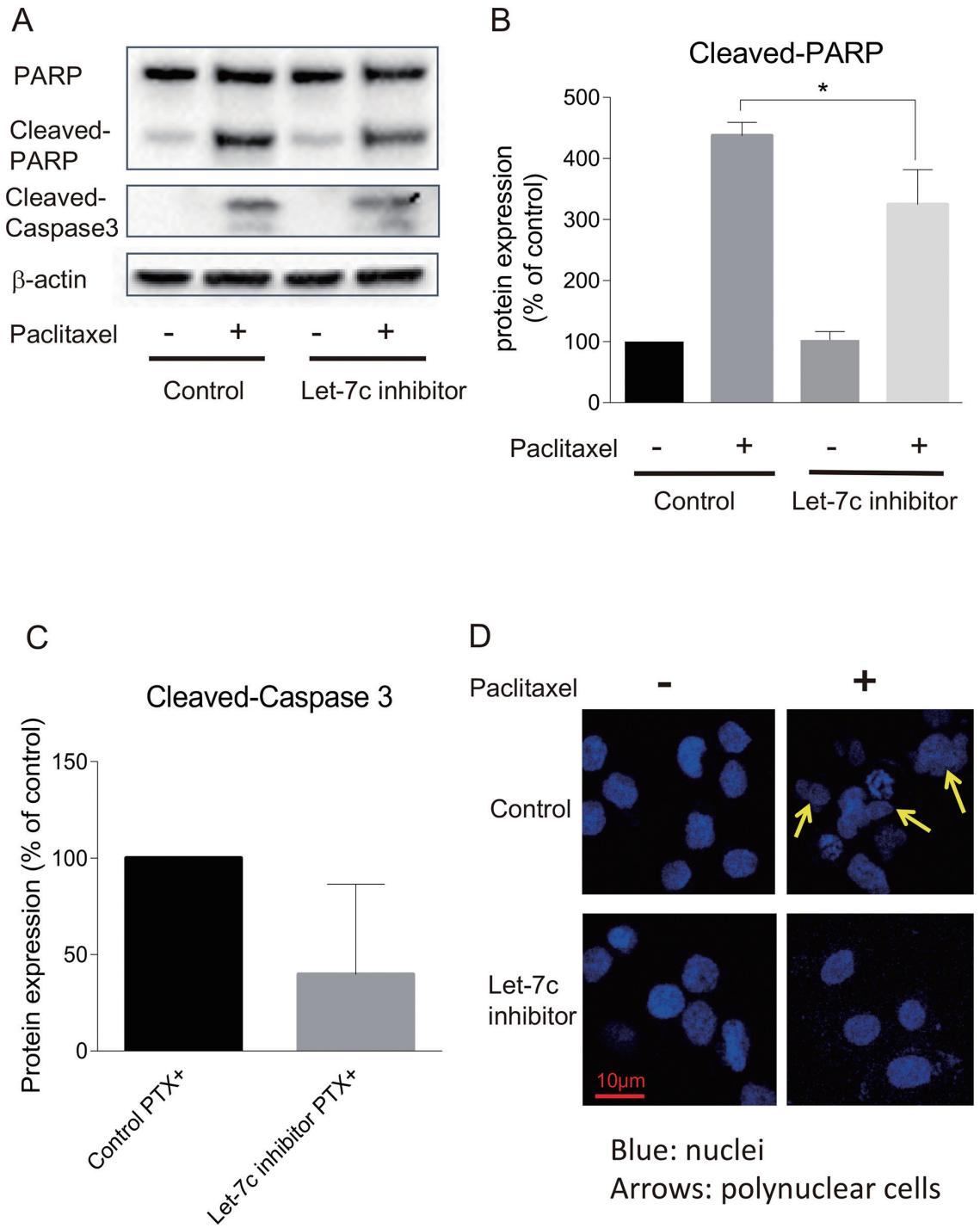


Fig. 3. Let-7c inhibition desensitized endometrial serous carcinoma cells to paclitaxel.

A. USPC1 cells were transfected with control or let-7c inhibitors for 24 h then treated with paclitaxel (5 nM) for 24 h. Extracted proteins were submitted to immunoblot analysis using antibodies specific for PARP, cleaved caspase-3, and *β*-actin. For the cropped blots, protein samples were run under the same conditional treatments and processed in parallel.

B. Quantified cleaved PARP protein levels. Results are representative of three independent experiments. Values represent the mean ± SEM.

*P < 0.05

C. Quantified cleaved Caspase3 protein levels. Results are representative of three independent experiments. Values represent the mean ± SEM. PTX: paclitaxel.

D. Nuclear USPC1 cell shapes were evaluated by immunofluorescence microscopy. USPC1 cells plated on glass-bottom 35 mm dishes were transfected with control or let-7c inhibitors for 24 h then treated with DMSO or paclitaxel (5 nM) for 24 h. After treatment, cells were stained with Hoechst 33342. Representative images are shown.

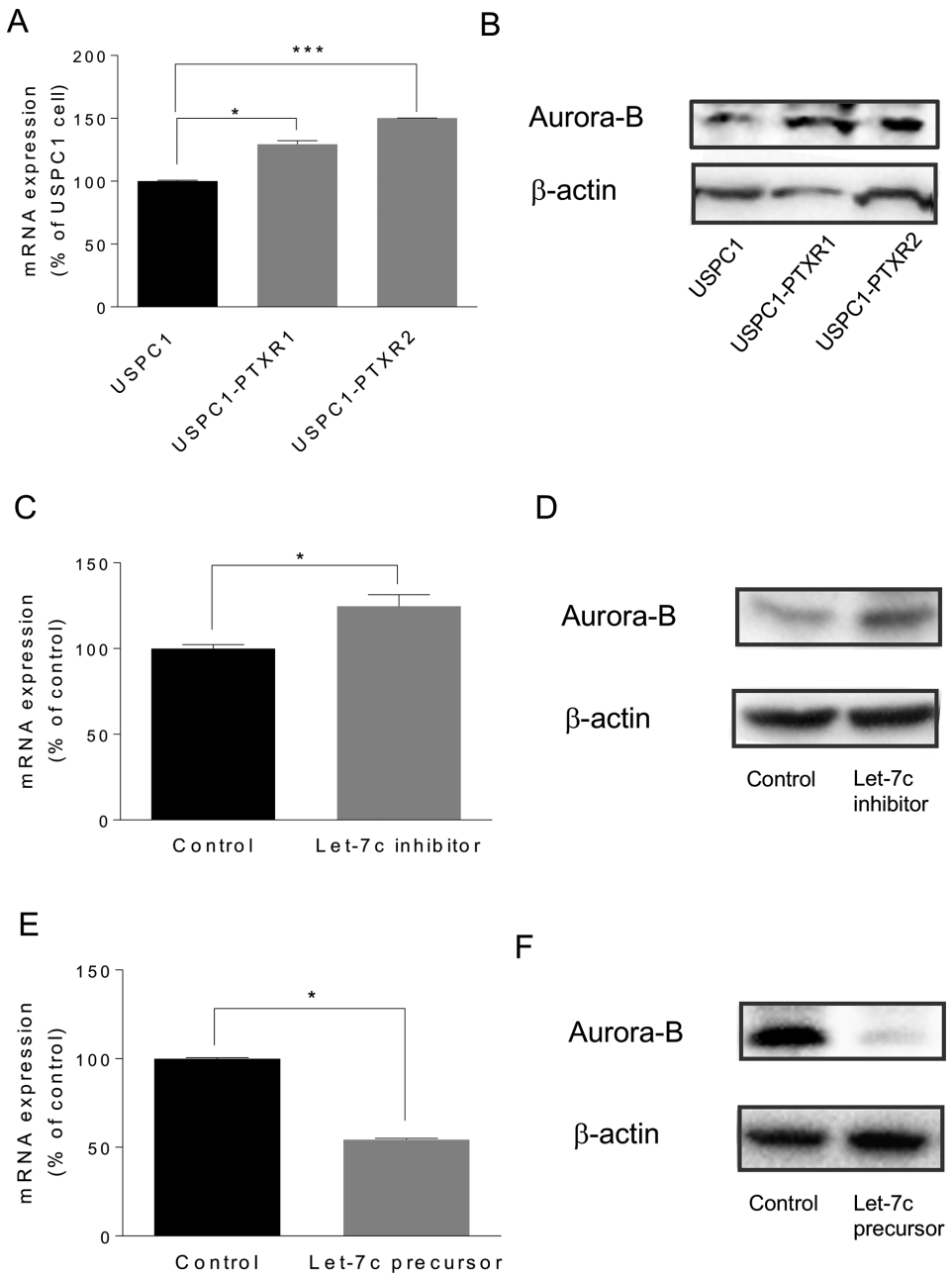


Fig. 4. Let-7c is involved in paclitaxel resistance inhibiting Aurora-B expression in endometrial serous carcinoma cells.
A. Aurora-B mRNA expression levels were determined using quantitative real-time PCR with a specific probe targeting Aurora-B mRNA. They were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels in USPC1, USPC1-PTXR1, and USPC1-PTXR2 cells.
B. Extracted proteins from USPC1, USPC1-PTXR1 and USPC1-PTXR2 cells were submitted to immunoblot analysis using antibodies specific for Aurora-B and *β*-actin.
C,D. USPC1 cells were transfected with control or let-7c inhibitor for 24 h followed by treatment with paclitaxel (5 nM). Protein and mRNA were then extracted. Aurora-B mRNA expression levels were determined using quantitative real-time PCR with a specific probe targeting Aurora-B mRNA. They were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels(C). Extracted proteins were submitted to immunoblot analysis using antibodies specific for Aurora-B and *β*-actin(D).
E, F. USPC1-PTXR1 cells were transfected with control or let-7c precursor for 24 h followed by treatment with paclitaxel (500 nM). Proteins and mRNAs were then extracted. Aurora-B mRNA expression levels were determined using quantitative real-time PCR with a specific probe targeting Aurora-B mRNA. They were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels (E). Extracted proteins were submitted to immunoblot analysis using antibodies specific for Aurora-B and *β*-actin(F).
For the cropped blots, protein samples were run under the same conditional treatments and processed in parallel. Values represent the mean ± SEM of three independent experiments.
*P < 0.05, ***P < 0.005.

other endometrial cancer subtypes (Chang-Halpenny et al. 2013). In this study, let-7c inhibition did not affect cell migration in USPC1 cells (data not shown). It seems that let-7c does not contribute to cell migration.

Zhan et al. (2013) reported that let-7c modulates cisplatin response in part by targeting ATP-binding cassette subfamily C member 2 (ABCC2). This protein transports various molecules across extra- and intra-cellular membranes. It is also a member of the MRP subfamily, which is involved in multi-drug resistance. It has been reported that ABCC2 correlates with cisplatin resistance (Surowiak et al. 2006) and paclitaxel resistance (Huisman et al. 2005). Notably, let-7c inhibition in USPC1 cells did not contribute to cisplatin sensitivity (Data not shown), and let-7c precursor treatment in USPC1 cells did not decrease ABCC2 mRNA expression in the current study (Data not shown). Therefore, let-7c does not appear to contribute to cisplatin and paclitaxel sensitivity in ESC via ABCC2. The effects of let-7c inhibition in USPC1 cells seems to be specific to paclitaxel.

Therapeutic small-interfering RNAs (siRNA) were granted FDA approval in 2018, yet microRNA drugs have not yet translated into FDA-approved candidates for medical intervention. However, candidate drugs are in clinical development and phase 1 and phase 2 clinical trials. Clinical utility in peripheral tissues for microRNA mimics that overexpress transcripts and microRNA repressors that silence transcript function have recently been reported (Hanna et al. 2019). Because microRNAs are smaller than proteins, they can be introduced to cells using techniques similar to those used for small siRNAs (Kota et al. 2009). In the first study to demonstrate let7's tumor-suppressor activity and potential therapeutic role in lung cancer, Esquela-Kerscher et al. (2008) reported that the let-7 microRNA reduces tumor mass in murine lung cancer.

Several earlier phase trials are currently investigating new microRNA drug candidates. For example, Regulus Therapeutics announced RGLS5579, which targets miR-10b, for potential trials in glioblastoma patients (Ghosh et al. 2018). Further improvement of microRNA delivery systems through the development of more specific carriers will enhance the specificity and efficiency of microRNA replacement therapy for cancers. With these advancements, microRNA let-7c precursor is expected to be a promising treatment option for paclitaxel-resistant ESC.

Acknowledgment

The authors thank Dr. A. Santin for providing cell line and Ms. E. Oba and Ms. N. Hashimoto for technical assistances.

Conflict of Interest

The authors declare no conflict of interest.

References

Al-Khafaji, A.S., Davies, M.P., Risk, J.M., Marcus, M.W., Koffa, M., Gosney, J.R., Shaw, R.J., Field, J.K. & Liloglou, T. (2017) Aurora B expression modulates paclitaxel response in non-small cell lung cancer. *Br. J. Cancer*, **116**, 592-599.

Barbuti, A.M. & Chen, Z.S. (2015) Paclitaxel through the ages of anticancer therapy: exploring its role in chemoresistance and radiation therapy. *Cancers* (*Basel*), **7**, 2360-2371.

Chang-Halpenny, C.N., Natarajan, S. & Hwang-Graziano, J. (2013) Early stage papillary serous or clear cell carcinoma confined to or involving an endometrial polyp: outcomes with and without adjuvant therapy. *Gynecol. Oncol.*, **131**, 598-603.

Cochrane, D.R., Spoelstra, N.S., Howe, E.N., Nordeen, S.K. & Richer, J.K. (2009) MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. *Mol. Cancer Ther.*, **8**, 1055-1066.

Esquela-Kerscher, A., Trang, P., Wiggins, J.F., Patrawala, L., Cheng, A., Ford, L., Weidhaas, J.B., Brown, D., Bader, A.G. & Slack, F.J. (2008) The let-7 microRNA reduces tumor growth in mouse models of lung cancer. *Cell Cycle*, **7**, 759-764.

Fader, A.N., Boruta, D., Olawaiye, A.B. & Gehrig, P.A. (2010) Uterine papillary serous carcinoma: epidemiology, pathogenesis and management. *Curr. Opin. Obstet. Gynecol.*, **22**, 21-29.

Fu, J., Bian, M., Jiang, Q. & Zhang, C. (2007) Roles of Aurora kinases in mitosis and tumorigenesis. *Mol. Cancer Res.*, **5**, 1-10.

Gascoigne, K.E. & Taylor, S.S. (2009) How do anti-mitotic drugs kill cancer cells? *J. Cell Sci.*, **122**, 2579-2585.

Ghosh, D., Nandi, S. & Bhattacharjee, S. (2018) Combination therapy to checkmate Glioblastoma: clinical challenges and advances. *Clin. Transl. Med.*, **7**, 33.

Grosshans, H., Johnson, T., Reinert, K.L., Gerstein, M. & Slack, F.J. (2005) The temporal patterning microRNA let-7 regulates several transcription factors at the larval to adult transition in C. elegans. *Dev. Cell*, **8**, 321-330.

Hanna, J., Hossain, G.S. & Kocerha, J. (2019) The potential for microRNA therapeutics and clinical research. *Front. Genet.*, **10**, 478.

Hosseinahli, N., Aghapour, M., Duijf, P.H.G. & Baradaran, B. (2018) Treating cancer with microRNA replacement therapy: a literature review. *J. Cell. Physiol.*, **233**, 5574-5588.

Huisman, M.T., Chhatta, A.A., van Tellingen, O., Beijnen, J.H. & Schinkel, A.H. (2005) MRP2 (ABCC2) transports taxanes and confers paclitaxel resistance and both processes are stimulated by probenecid. *Int. J. Cancer*, **116**, 824-829.

Ishibashi, M., Toyoshima, M., Zhang, X., Hasegawa-Minato, J., Shigeta, S., Usui, T., Kemp, C.J., Grandori, C., Kitatani, K. & Yaegashi, N. (2018) Tyrosine kinase receptor TIE-1 mediates platinum resistance by promoting nucleotide excision repair in ovarian cancer. *Sci. Rep.*, **8**, 13207.

Johnson, C.D., Esquela-Kerscher, A., Stefani, G., Byrom, M., Kelnar, K., Ovcharenko, D., Wilson, M., Wang, X., Shelton, J., Shingara, J., Chin, L., Brown, D. & Slack, F.J. (2007) The let-7 microRNA represses cell proliferation pathways in human cells. *Cancer Res.*, **67**, 7713-7722.

Johnson, S.M., Grosshans, H., Shingara, J., Byrom, M., Jarvis, R., Cheng, A., Labourier, E., Reinert, K.L., Brown, D. & Slack, F.J. (2005) RAS is regulated by the let-7 microRNA family. *Cell*, **120**, 635-647.

Jordan, M.A., Thrower, D. & Wilson, L. (1991) Mechanism of inhibition of cell proliferation by Vinca alkaloids. *Cancer Res.*, **51**, 2212-2222.

Kitatani, K., Usui, T., Sriraman, S.K., Toyoshima, M., Ishibashi, M., Shigeta, S., Nagase, S., Sakamoto, M., Ogiso, H., Okazaki, T., Hannun, Y.A., Torchilin, V.P. & Yaegashi, N. (2016) Ceramide limits phosphatidylinositol-3-kinase C2beta-

controlled cell motility in ovarian cancer: potential of ceramide as a metastasis-suppressor lipid. *Oncogene*, **35**, 2801-2812.

Kota, J., Chivukula, R.R., O'Donnell, K.A., Wentzel, E.A., Montgomery, C.L., Hwang, H.W., Chang, T.C., Vivekanandan, P., Torbenson, M., Clark, K.R., Mendell, J.R. & Mendell, J.T. (2009) Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell*, **137**, 1005-1017.

Kumar, M.S., Lu, J., Mercer, K.L., Golub, T.R. & Jacks, T. (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat. Genet.,* **39**, 673-677.

Liu, P., Qi, M., Ma, C., Lao, G., Liu, Y., Liu, Y. & Liu, Y. (2013) Let7a inhibits the growth of endometrial carcinoma cells by targeting Aurora-B. *FEBS Lett.*, **587**, 2523-2529.

Mulholland, E.J., Green, W.P., Buckley, N.E. & McCarthy, H.O. (2019) Exploring the potential of MicroRNA let-7c as a therapeutic for prostate cancer. *Mol. Ther. Nucleic Acids*, **18**, 927-937.

Noujaim, M., Bechstedt, S., Wieczorek, M. & Brouhard, G.J. (2014) Microtubules accelerate the kinase activity of Aurora-B by a reduction in dimensionality. *PloS one*, **9**, e86786.

Pritchard, C.C., Cheng, H.H. & Tewari, M. (2012) MicroRNA profiling: approaches and considerations. *Nat. Rev. Genet.*, **13**, 358-369.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R. & Ruvkun, G. (2000) The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. *Nature*, **403**, 901-906.

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H. & Bartel, D.P. (2006) Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. *Cell*, **127**, 1193-1207.

Si, W., Shen, J., Zheng, H. & Fan, W. (2019) The role and mechanisms of action of microRNAs in cancer drug resistance. *Clin. Epigenetics*, **11**, 25.

Siegel, R.L., Miller, K.D. & Jemal, A. (2019) Cancer statistics, 2019. *CA Cancer J. Clin.*, **69**, 7-34.

Surowiak, P., Materna, V., Kaplenko, I., Spaczynski, M., Dolinska-Krajewska, B., Gebarowska, E., Dietel, M., Zabel, M. & Lage, H. (2006) ABCC2 (MRP2, cMOAT) can be localized in the nuclear membrane of ovarian carcinomas and correlates with resistance to cisplatin and clinical outcome. *Clin. Cancer Res.*, **12**, 7149-7158.

Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y., Mitsudomi, T. & Takahashi, T. (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.*, **64**, 3753-3756.

Tang, H., Ma, M., Dai, J., Cui, C., Si, L., Sheng, X., Chi, Z., Xu, L., Yu, S., Xu, T., Yan, J., Yu, H., Yang, L., Kong, Y. & Guo, J. (2019) miR-let-7b and miR-let-7c suppress tumourigenesis of human mucosal melanoma and enhance the sensitivity to chemotherapy. *J. Exp. Clin. Cancer Res.*, **38**, 212.

Xie, Y., Zhang, H., Guo, X.J., Feng, Y.C., He, R.Z., Li, X., Yu, S., Zhao, Y., Shen, M., Zhu, F., Wang, X., Wang, M., Balakrishnan, A., Ott, M., Peng, F., et al. (2018) Let-7c inhibits cholangiocarcinoma growth but promotes tumor cell invasion and growth at extrahepatic sites. *Cell Death Dis.*, **9**, 249.

Yin, S., Bhattacharya, R. & Cabral, F. (2010) Human mutations that confer paclitaxel resistance. *Mol. Cancer Ther.*, **9**, 327-335.

Zhan, M., Qu, Q., Wang, G. & Zhou, H. (2013) Let-7c sensitizes acquired cisplatin-resistant A549 cells by targeting ABCC2 and Bcl-XL. *Pharmazie*, **68**, 955-961.

Zhang, G.M., Long, X.H., Liu, J.M., Zhu, L.B., Chen, X.Y., Huang, S.H., Zhang, Z.H. & Liu, Z.L. (2015) Let-7i inhibits the malignant phenotype of osteosarcoma cells by targeting Aurora-B. *Mol. Med. Rep.*, **12**, 3543-3548.

Zhu, X., Wu, L., Yao, J., Jiang, H., Wang, Q., Yang, Z. & Wu, F. (2015) MicroRNA let-7c inhibits cell proliferation and induces cell cycle arrest by targeting CDC25A in human hepatocellular carcinoma. *PLoS One*, **10**, e0124266.