MicroRNA MiR-214 Regulates Ovarian Cancer Cell Stemness by Targeting p53/Nanog*

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Significance:

Conclusion:

Expression of miR-214 induces, whereas knockdown of miR-214 decreases, OCSC and Nanog. MiR-214 targets p53, a repressor of Nanog.

Background:

Ovarian cancer stem cells (OCSC) play a critical role in chemoresistance and relapse.

Results:

Expression of miR-214 induces, whereas knockdown of miR-214 decreases, OCSC and Nanog. MiR-214 targets p53, a repressor of Nanog.

Conclusions:

MiR-214 is a target for OCSC.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, Cell Culture, and Transfection—Expression plasmids of p53 and miR-214 have been previously described (2, 18). pMIR-p53 and MUT-pMIR-p53 were created by ligation of the wild-type and mutant miR-214 binding motif of p53-3’ UTR (746 nt) into the MluI/BamHI sites of pMIR-REPORT vector (Ambion), respectively. Nanog promoter-driven GFP was generated by cloning 806 bp upstream of the Nanog transcriptional starting site, which contains the p53 binding site, into the XhoI/Sacl sites of the phrGFP promoter-less vector (Stratagene).

Anti-p53 and -MDM2 antibodies were from Santa Cruz Biotechnology, Inc. Antibodies against BAX, Bmi-1, Sox2, Oct4,
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Nanog, cleaved poly (ADP-ribose) polymerase (PARP), and cleaved caspase 3 were purchased from Cell Signaling Technology, Inc.

The ovarian cancer cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37 °C. Transfections used either siPORT NeoFX transfection agent (Ambion) for oligonucleotides or Lipofectamine 2000 (Invitrogen) for the expression plasmid.

Cell Proliferation and Cell Survival—Cells were plated in 96-well cell culture plates (1 x 10⁴ cells/well) and transfected with indicated oligonucleotides and plasmids. Following incubation for 24 h, cells were treated with or without cisplatin (CDDP, Sigma) or doxorubicin (Sigma) for 24 h. Cell growth and survival were examined with cell counting and an MTT assay according to the instructions of the manufacturer (Sigma). In addition, the programmed cell death was determined by cleavage of PARP and caspase 3.

Quantitative RT-PCR—Total RNA was extracted from cells with TRIzol (Invitrogen) agent according to the instructions of the manufacturer. Reverse transcription and real-time PCR were performed with The TaqMan® MicroRNA reverse transcription kit and TaqMan Universal PCR Master Mix (Applied Biosystems), respectively, using Ambion miRNA primers. The results were calculated and normalized to a control gene (RNU6B). The mRNA expression levels of p53 and Nanog were measured by semiquantitative RT-PCR. The primer sequences are as follows: p53 (sense), 5’-TTGGATCATGTTTCTCTGGCC; p53 (antisense), 5’-TTGAATTCAGGCTCCCCTTT-; Nanog (sense), 5’-ATGCTTTGTGCCACACCTTTCC; Nanog (antisense), 5’-GCCAGTTGTT-; p53 (antisense), 5’-TTGAATTCAGGCTCCCCTTT-; Nanog (sense), 5’-ATGCTTTGTGCCACACCTTTCC; Nanog (antisense), 5’-GCCAGTTGTT-

Luciferase Assay—Cells were transfected with different reporter plasmids, 0.1 μg miR-214 or control plasmid, and 0.05 μg reporter plasmid. After 48 h of incubation, cells were subjected to a luciferase reporter assay or control/well on 96-well plates. Following 48 h of miR-214 b l e , 5n mo f miR-214 /H11032 GCC-3 /H11032- ATGCCTGTGATTTGTGG-; Nanog (sense), 5’CTTGCG-3’ GGCC; p53 (antisense), 5’-TTGAATTCAGGCTCCCCTTT-; Nanog (sense), 5’-ATGCTTTGTGCCACACCTTTCC; Nanog (antisense), 5’-GCCAGTTGTT-

 Luciferase Assay—Cells were transfected with different reporter plasmids, 0.1 μg miR-214 or control plasmid, and 0.05 μg reporter plasmid. After 48 h of incubation, cells were subjected to a luciferase reporter assay using the luciferase assay system. Luciferase activities were normalized by β-galactosidase activities. Each experiment was repeated at least three times in triplicate.

ALDEFLUOR Assay and Sphere Growth—ALDH1 activity was detected using the ALDEFLUOR assay kit (Stemcell Technologies) as described by the manufacturer (19). Briefly, cells were suspended in ALDEFLUOR assay buffer containing an ALDH1 substrate, bodipy-aminoacetaldehyde, at 1.5 μM and incubated for 1 h at 37 °C. A specific inhibitor of ALDH1, diethylaminoalkylaldehyde, at a 10-fold molar excess, was used as a negative control. Flow cytometry data were analyzed by BD FACSDiva software version 6.1.3 (BD Biosciences) or FlowJo software (TreeStar).

Sphere culture was carried out as described previously (19). Briefly, cells were plated in ultra-negative attachment 6-well plates (Corning) at a density of 5000 viable cells/well. Cells were grown in a serum-free sphere culture medium (MammoCult, StemCell Technologies) supplemented with MammolCult proliferation supplements for 12 days. Sphere numbers were counted under microscopy.

Western Blot and Immunofluorescence—A Western blot analysis was performed as described previously (18). For immuno

RESULTS

MiiR-214 Regulates Ovarian Cancer Stem-like Cells—The findings that miR-214 plays a critical role in chemoresistance and metastasis in ovarian cancer and melanoma (2, 5) prompted us to examine the role of miR-214 in regulating OCSC. Supplemental Fig. S1 shows miR-214 levels and p53 status in a panel of ovarian cancer cell lines and two immortalized ovarian surface epithelial cell lines, T80 and MCC3-HOSE. Because the endogenous miR-214 level is high in A2780S and OV8 and low in OV2008 and OV3 cells, we knocked down miR-214 in these cells and ectopically expressed miR-214 in wild-type p53 OV8 cells (Fig. 1A). OCSCs were detected by ALDEFLUOR assay, and sorting of the cells labeled by ALDH1, a common marker of OCSC (19–21). Fig. 1A shows that depletion of miR-214 reduced the ALDEFLUOR signal in wild-type p53 A2780S but not in p53-mutant OV8 cells. Enforcing expression of miR-214 significantly increased OCSC in wild-type p53 OV2008 but not in p53-mutant OV8 cells (supplemental Fig. S2). Furthermore, sphere growth was evaluated in these cell lines. We observed that knockdown of miR-214 decreased, whereas ectopic expression of miR-214 enhanced, sphere growth in A2780S/OV8 and OV2008/OV10 cells, respectively, with minimal effect on OV8 and SKOV3 cells (Fig. 1D and supplemental Fig. S3). Because CSCs closely relate to chemoresistance (22), we further investigated miR-214 effects on chemosensitivity of OCSC. As shown in supplemental Fig. S4, ALDH1-positive A2780S and OV8 cells expressed high levels of miR-214 and resisted cisplatin and doxorubicin treatment when compared with ALDH1-negative cells. Knockdown of miR-214 in ALDH1-positive cells largely restored their sensitivity to chemotherapeutic agents. These results suggest that miR-214 regulates OCSC in a subset of ovarian cancers.

Nanog Is Regulated by miR-214 in a Subset of Ovarian Cancers—Because accumulating evidence indicates that transcription factors Nanog, Sox-2, and Oct-4 and polycomb ring finger oncogene Bmi-1 are essential to maintain the pluripotent stem cell phenotype (17, 23), we examined the effect of miR-214 on their expression. Western blot analysis and semiquantitative RT-PCR revealed that protein and mRNA levels of Nanog were decreased in wild-type p53 A2780S and OV8 cells (supplemental Fig. S4). Knockdown of miR-214 decreased Nanog expression in wild-type p53 A2780S and OV10 cells (Fig. 2B) but not in p53-mutant OV8 and A2780CP cells (supplemental Fig. S5B). We did not observe significant
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Identification of p53 as a Direct Target of miR-214—Because miRNAs negatively regulate their target genes (24), Nanog would not be a direct target of miR-214. Because miR-214 induces Nanog expression at the mRNA level, miR-214 could target a transcription factor(s) that represses Nanog transcription. Previous studies have shown that Nanog is negatively regulated by p53 and positively regulated by Oct4 and Sox2 (15, 25, 26). Our findings that miR-214 regulates OCSC preferentially in wild-type p53 (OV2008, OV433, A2780, and OV10) but not in p53-mutant (SKOV3, OV3, OV8, and A2780CP) cells (27, 28) prompted us to examine whether miR-214 regulates p53.

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Because OV2008 and OV433 cells express a low level of miR-214, we transfected the cells with pre-miR-214. This resulted in a reduction of about 60–70% of p53 protein and mRNA levels (Fig. 4, A–C). In contrast, A2780 and OV10 cells express a high level of miR-214, so we proceeded to suppress miR-214 with miR-214 ASO. This resulted in an increase in protein and mRNA levels of p53 and its downstream targets MDM2 and BAX (supplemental Fig. S8). Ectopic expression of miR-214 induced Nanog is mediated by p53. An 806-bp fragment of the Nanog promoter region, which contains the p53 binding site, was cloned into the pGL3-basic and phrGFP promoter-less vectors (Fig. 6A). A luciferase assay revealed that the basal level of Nanog promoter activity is much lower in wild-type p53 OV429 and OV10 than in p53-mutant OV8 and A2780CP cells (Fig. 6B). Ectopic expression of miR-214 induced, whereas knockdown of miR-214 decreased, Nanog promoter activity in wild-type p53 but not p53-mutant cells (Fig. 6C). Moreover, Nanog promoter-driven GFP expression was increased upon expression of miR-214 and was decreased following miR-214 knockdown in OV429 and OV10 but not OV8 and A2780CP cells (Fig. 6D). Accordingly, inverse expression of p53 and Nanog at mRNA and protein levels was detected in miR-214-manipulated OV429 and OV10 cells (Fig. 6E and supplemental Fig. S8). In addition, ectopic expression of p53 without the 3′ UTR and knockdown of p53 abrogated the effects of miR-214 on Nanog expression (Fig. 6F). Taken together, these data indicate that miR-214 regulates Nanog expression via targeting p53.

WithDRAWN
Expression of p53 Overrides miR-214-induced OCSC and Chemoresistance—Having demonstrated that p53 is a direct target of miR-214, we also examined whether expression of p53 will override the effect of miR-214 on OCSC property and chemoresistance. Stable miR-214/OV2008 clonal cells were transfected with p53 cDNA without the 3’/H11032 UTR. Vector-transfected cells were used as a control (Fig. 7A). As shown in Fig. 7B, expression of p53 abrogated the miR-214-enhanced OCSC population. Furthermore, expression of miR-214 decreased, whereas knockdown of miR-214 increased, CDDP-induced cleavage of PARP and caspase 3 as well as cell death (Figs. 7, C and D). However, coexpression of p53 significantly reduced the miR-214 protective effect on cell death induced by CDDP (Fig. 7E). These data further support the finding of p53 as a direct target of miR-214 and suggest miR-214 regulation of OCSC and chemoresistance, at least to some extent, through targeting p53.

DISCUSSION

Up-regulation of miR-214 has been detected in various human malignancies, including pancreatic, prostate, gastric, breast, and ovarian cancers as well as malignant melanoma (2–8). Furthermore, miR-214 has been shown to play an important role in chemoresistance, tumor progression, and metastasis (2, 5). In this study, we demonstrated that miR-214 regulates ovarian cancer stem cells. Enforcing expression of miR-214 induces, whereas depletion of miR-214 decreases, OCSC properties as well as expression of Nanog. MiR-214 represses p53 by directly interacting with the 3’ UTR of p53. Moreover, we showed that p53 mediates miR-214-induced Nanog, OCSC, and chemoresistance. These findings are important for several reasons. First, they provide a mechanistic understanding of the miR-214 function in OCSC and chemoresistance. Second, these data further support the notion that miR-214 is an oncomiR in human ovarian cancer. Finally, restoration of p53 by inhibition of miR-214 could be a valuable therapeutic approach in ovarian cancer.

Previous studies have shown that miR-214 is deregulated in CSC and high metastatic tumors (11, 30, 31) and that miR-214 induces cell migration and invasion and chemoresistance (2, 5), which are characteristics of CSC (10). These findings implicate an important role of miR-214 in the regulation of CSC. ALDH1 has been proved to be a useful marker for cancer stem cells and has been widely used to isolate CSC in various malignancies, including ovarian cancer (11, 19–21). In ovarian carcinoma, ALDH1-positive cells exhibit sphere growth, highly tumorigenicity, and resistance to chemotherapy (19, 32–34). Moreover, the expression level of ALDH1 has been correlated with a poor prognosis in serous ovarian cancers (19). We showed in this
study that ALDH1-positive ovarian cancer cells express high levels of miR-214 (supplemental Fig. S4A) and CSC markers, including LIN28, Nanog, OCT4, and SOX2 (supplemental Fig. S9). Moreover, we demonstrated that knockdown of miR-214 reduced Nanog expression, ALDH1-positive cell population, OCSC growth, and sensitization of OCSC cells to therapeutic agent-induced apoptosis. In contrast, expression of miR-214 had the opposite effect. Thus, we provide direct evidence that...
miR-214 plays a critical role in maintaining OCSC properties and that miR-214 is a critical therapeutic target in ovarian cancer.

The p53 level, in addition to mutation, has been linked to control of stem cell and CSC properties. Loss of p53 diminishes spontaneous apoptosis and differentiation of embryonic stem cells. Several studies have shown that loss of p53 improves the generation of induced pluripotent stem cells from adult cells (35–38). In hematopoietic stem cells, p53 negatively regulates their self-renewal (39). Mice deficient in p53 show enhanced HSC self-renewal and have an increased HSC pool size. Similarly, loss of p53 has been shown to increase CSC self-renewal, and expression of p53 represses CSC properties (40). Furthermore, p53 has been shown to repress expression of Nanog, a key molecule in maintaining pluripotency and self-renewal of stem cells, through binding and inhibiting its promoter (25, 41). Because miRNA functions as a regulator of gene expression, it is plausible that miRNA plays an important influence on stem cells through modulation of p53. In fact, a recent study showed that miR-33 enhances HSC transplantation efficiency through down-regulation of p53 (42). We showed in this study that miR-214 represses p53 in ovarian cancer cells expressing wild-type or mutant p53 (Fig. 3 and supplemental Fig. S10). However, miR-214 had no effect on Nanog expression in p53-mutant cells (supplemental Fig. S10) because of the fact that mutated p53 loses its DNA-binding activity (43). Our data showed that miR-214 regulates OCSC primary in wild-type p53 cells even though a number of protein-coding genes such as PTEN, TFAP2C, Sufu, and Patched (PTCH) are targeted by miR-214 (2, 5, 44, 45). Nevertheless, these findings further suggest the impor-

FIGURE 7. Expression of p53 abrogated the effects of miR-214 on OCSC property and chemoresistance. OV2008 cells were transfected with miR-214 together with and without p53 cDNA and then immunoblotted with the indicated antibodies (A), ALDEFLUOR, and sphere growth assay (B). SSC, side-scattered light; Ctr, control. C–E, A2780S and OV2008 cells were transfected with the indicated oligos and plasmid. After 24 h of CDDP treatment, cleaved PARP, cleaved caspase-3, and cell viability were examined. Experiments were repeated three times in triplicate. Note: $p < 0.05$ when comparing the untreated group with the CDDP, CDDP/pre-miR-214, CDDP/ASO miR-214, and CDDP/pre-miR-214/p53 groups. In addition, it is also statistically significant ($p < 0.05$) of CDDP versus CDDP/ASO miR-214 and CDDP versus CDDP/pre-miR-214/p53.

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WITHDRAWN
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Supplemental Data

**Figure S1. MiR-214 expression levels and p53 status in ovarian cell lines.** (A) qRT-PCR analysis was performed with specific miR-214 primers in indicated cell lines. (B) The list of p53 status in ovarian cancer cell lines.

**Figure S2. MiR-214 regulates OCSC in wild-type p53 ovarian cancer cells.** (A - C) Indicated cell lines were transfected with pre-miR-214 or ASO miR-214 and control oligo. After 72 h of transfection, cells were subjected to CD133 labeling (upper panels of A), qRT-PCR analysis of miR-214 expression level (bottom panels of A) and ALDEFLUOR analysis (B, C).

**Figure S3. MiR-214 does not affect sphere growth in mutant p53 cells OV8 and SKOV3.** Cells were plated in ultra-negative attachment 6-well plate (5,000 viable cells/well) and were grown in a serum-free sphere culture medium for 12 days. Sphere numbers were counted under microscopy.

**Figure S4. Knockdown of miR-214 overcomes chemoresistance in ALDH1-positive cells.** ALDH1-positive and -negative cells were isolated from A2780S and OV2008 cells by flow sorting and were analyzed for miR-214 levels. ALDH1-positive cells were transfected with indicated oligos, cells were treated with or without CDDP (B) and doxorubicin (C). After 24 h of treatment, cells were subjected to MTT assay. Data shown are mean ± SD for each experimental group and all the experiments were repeated 3 times in triplicate.

**Figure S5. MiR-214 does not regulate Nanog in mutant p53 cells.** Indicated cells were transfected with pre-miR-214 (A) or ASO miR-214 (B) as well as control oligo. Following incubation of 72 h, cells were subjected to immunoblot and RT-PCR analyses.

**Figure S6. P53 expression is negatively regulated by miR-214.** (A) qRT-PCR and Western blot analysis show inverse correlation of miR-214 and p53 levels in a majority ovarian cancer cell lines examined. (B) OV2008 and A2780 cells were transfected with indicated oligos and then immuno-stained with p53 antibody after 48 h of transfection.

**Figure S7. MiR-214 represses the expression of p53 and its downstream targets.** OVCA433 cells were infected with lenti-miR-214 and lenti-miR vectors. After selection with puromycin, 3 stable miR-214 clonal cell lines were subjected to immunoblot analysis with indicated antibodies.

**Figure S8. Nanog is regulated by miR-214 in wild-type p53 cells.** OV10 cells were transfected with indicated oligos and then subjected to RT-PCR (upper panels) and immunoblot analyses (bottom panels).

**Figure S9. ALDH-positive cells express CSC markers.** ALDH-negative and – positive cells were sorted and then immunoblotted with indicated antibodies.
Figure S10. MiR-214 represses p53 but does not induce Nanog expression in p53-mutant cells. OV3 and A2780CP cells were transfected with pre-miR-214 (left) and ASO miR-214 (right), respectively. After incubation for 72 h, cells were analyzed by immunoblot (upper panels) and semi-quantitative RT-PCR (bottom panels) for p53 and Nanog expression.
### Table

| Cell line   | p53 status |
|-------------|------------|
| OV10        | Wild type  |
| A2780CP     | Mutation   |
| OV B2       | Wild type  |
| OV433       | Wild type  |
| OV2008      | Wild type  |
| OV429       | Wild type  |
| A2780S      | Wild type  |
| OV8         | Wild type  |
| OV3         | Mutation   |
| SKOV3       | Wild type  |
| T80         | Wild type  |
| PEO1        | Mutation   |
| C13         | Wild type  |
| OV2008      | Wild type  |
| MCC3-HOSE   |            |

**Figure S1**
Figure S2
Figure S3

(OV433)

Ctr. Oligo pre-miR-214

No. of Spheres/5,000 cells

p=0.02

(OV10)

Ctr. Oligo ASOmiR-214

No. of Spheres/5,000 cells

p=0.04

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Figure S3
Figure S4
A

|       | Ctr. Oligo | pre-miR-214 | Ctr. Oligo | pre-miR-214 |
|-------|------------|-------------|------------|-------------|
| Nanog | 1.0        | 1.2         | 1.0        | 0.9         |
| Oct-4 |            |             |            |             |
| Sox-2 |            |             |            |             |
| Bmi-1 |            |             |            |             |
| Actin |            |             |            |             |

B

|       | Ctr. Oligo | ASO miR-214 | Ctr. Oligo | ASO miR-214 |
|-------|------------|-------------|------------|-------------|
| Oct-4 | 1.0        | 1.2         | 1.0        | 1.3         |
| Sox-2 |            |             |            |             |
| Bmi-1 |            |             |            |             |
| Actin |            |             |            |             |
| Nanog | 1.0        | 1.1         | 1.0        | 1.3         |
| Actin |            |             |            |             |
Figure S6

A

-\(\Delta Ct\) vs. Cell Lines

**Figure S6A**

Bar graph showing -\(\Delta Ct\) values for various cell lines. The x-axis represents different cell lines, and the y-axis represents -\(\Delta Ct\) values.

B

Immunoblot analysis of p53 and Actin expression in different cell lines.

**Figure S6B**

Immunofluorescence images showing the effect of Ctr. Oligo and ASO miR-214 on A2780S and OV2008 cells. The images indicate changes in cellular structures under different conditions.

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Figure S7
Figure S8
Figure S9

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Figure S10

CTR. OLIGO PRE-miR214

CTR. OLIGO ASO miR214

p53

Nanog

Actin

protein

mRNA

(OV3) (A2780CP)

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MicroRNA MiR-214 Regulates Ovarian Cancer Cell Stemness by Targeting p53/Nanog
Cheng-Xiong Xu, Meng Xu, Lei Tan, Hua Yang, Jennifer Permuth-Wey, Patricia A. Kruk, Robert M. Wenham, Santo V. Nicosia, Johnathan M. Lancaster, Thomas A. Sellers and Jin Q. Cheng

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