Ovarian cancer is the leading cause of death among all gynecological malignancies due to the development of acquired chemoresistance and disease relapse. Although the role of cancer stem cells (CSCs), a subset of tumor cells with the self-renewal and differentiation capabilities, in therapeutic resistance is beginning to be better understood, the significance of epigenetic regulatory mechanisms responsible for integrating the stemness with drug resistance remain poorly understood. Here we identified that lysine demethylase KDM3A as a critical regulator of ovarian cancer stemness and cisplatin resistance by inducing the expressions of pluripotent molecules Sox2 and Nanog and anti-apoptotic B-cell lymphoma 2 (Bcl-2), respectively. In addition, KDM3A induces ovarian cancer growth while antagonizing cellular senescence by repressing the expression of cyclin-dependent kinase inhibitor, p21Waf1/Cip1. The underlying mechanism of the noted biological processes include KDM3A-mediated stimulation of Sox2 expression, and demethylating p53 protein and consequently, modulating its target genes such as Bcl-2 and p21Waf1/Cip1 expression. Consistently, KDM3A depletion inhibited the growth of subcutaneously implanted cisplatin-resistant human ovarian cancer cells in athymic nude mice. Moreover, KDM3A is abundantly expressed and positively correlated with Sox2 expression in human ovarian cancer tissues. In brief, our findings reveal a novel mechanism by which KDM3A promotes ovarian CSCs, proliferation and chemoresistance and thus, highlights the significance of KDM3A as a novel therapeutic target for resistant ovarian cancer.

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RESULTS
KDM3A is highly expressed in cisplatin-resistant ovarian cancer cells
To explore the epigenetic mechanisms of cisplatin resistance in ovarian cancer, we screened the expression of JMJD family histone demethylases in parental (OVCAR-5) and cisplatin-resistant (OVCAR-5/CDDP) ovarian cancer cell lines by real-time reverse transcriptase (RT)-PCR. As shown in Figure 1a, our screening identified that KDM3A, KDM4D and PHF2 were highly expressed in OVCAR-5/CDDP cells as compared with OVCAR-5 cells. Emerging evidences suggested that KDM3A is a hypoxic target gene and mediates the hypoxia-inducible factor-1α-induced tumor progression through epigenetic mechanisms.16 Since it is well established that hypoxic tumor microenvironment drives the aggressiveness and chemoresistance in ovarian cancer,17,18 we focused our further studies on KDM3A. Next, we examined the protein expression of KDM3A in parental and cisplatin-resistant OVCAR-5 cells by immunoblotting. Consistent with the mRNA expression, OVCAR-5/CDDP cells highly expressed KDM3A protein as compared with parental cells (Figure 1b). Further, to check whether the high abundance of KDM3A correlated with platinum resistance in other ovarian cancer cells, we determined the KDM3A protein expression in parental and platinum-resistant SKOV3 and A2780 cells. Notably, all the cisplatin-resistant ovarian cancer cells consistently overexpressed KDM3A protein as compared with parental cell line (Figures 1b-d). Altogether, these results indicated that KDM3A might be a crucial epigenetic factor required for platinum resistance in ovarian cancer.

KDM3A depletion inhibited cell cycle progression by inducing G2/M arrest
To understand the functional role of KDM3A in resistant ovarian cancer, we stably knocked down KDM3A by lentiviral method in OVCAR-5/CDDP and A2780/CDDP cells by using two shRNAs targeting different sequences on KDM3A mRNA. As demonstrated by immunoblot, both the KDM3A shRNAs efficiently depleted KDM3A protein in OVCAR-5/CDDP and A2780/CDDP cells (Figures 2a and b). Surprisingly, we observed that significant proportion of KDM3A-depleted OVCAR-5/CDDP and A2780/CDDP cells became large, flat and vacuolated (Figure 2c). Moreover, our microscopic observation also revealed that KDM3A-depleted OVCAR-5/CDDP and A2780/CDDP cells grew relatively slower than scrambled control cells with high abundance of floating cell population. Taken together, these observations suggest that KDM3A might play a key role in ovarian cancer growth and survival. To check this possibility, we evaluated the cell cycle profile of OVCAR-5/CDDP and A2780/CDDP cells expressing scrambled and KDM3A shRNAs by flow cytometry. Interestingly, KDM3A depletion led to G2/M cell cycle arrest, which was accompanied by increased apoptosis (Figures 2d and e, 3e and f), indicating that KDM3A is crucial for ovarian cancer cell proliferation and survival.

KDM3A promotes ovarian cancer growth and survival by inhibiting cellular senescence and apoptosis
Cellular senescence, a state of irreversible growth arrest, which plays a major role in aging and cancer is often accompanied by morphological changes such as cells becoming large, flat and multinucleated.19 Since we observed that growth arrest was accompanied by the presence of large, flat and vacuolated cells in KDM3A knockdown cells, we stained for senescence-associated β-galactosidase activity, a hallmark of cellular senescence. Indeed, both OVCAR-5/CDDP and A2780/CDDP cells expressing KDM3A shRNAs showed high abundance of senescence-associated β-galactosidase positive cells as compared with scrambled control cells (Figures 3a-d), indicating that KDM3A prevents the cellular senescence to promote sustained growth of ovarian cancer. Because we noticed a high proportion of floating cell population in KDM3A-depleted wells, we quantified the percentage of apoptotic cells by flow cytometry. As expected, KDM3A depletion inhibited in vivo growth of ovarian cancer xenograft in mice and abundantly expressed in human ovarian cancer tissues.
Figure 2. KDM3A knockdown induces cell cycle arrest in cisplatin-resistant ovarian cancer cells. (a and b) Immunoblot analysis of KDM3A expression in OVCAR-5/CDDP (a) and A2780/CDDP (b) cells expressing Scr and KDM3A shRNAs. (c) Photomicrograph showing the morphological changes in OVCAR-5/CDDP and A2780/CDDP cells following KDM3A knockdown. The giant vacuolated cells are indicated by arrows. (d) Cell cycle profile of OVCAR-5/CDDP cells expressing Scr and KDM3A shRNA as determined by flow cytometry. (e) Cell cycle profile of A2780/CDDP cells expressing Scr and KDM3A shRNAs as determined by flow cytometry. Each bar represents mean ± s.d. of triplicate samples from representative experiments. *P < 0.05, Student’s t-test.

KDM3A knockdown significantly increased the apoptotic cell death in OVCAR-5/CDDP and A2780/CDDP cells (Figures 3e and f). Further, to confirm the KDM3A shRNAs-induced apoptosis, we also examined the expressions of classical apoptotic markers such as cleaved PARP and caspase-7 by immunoblotting. In line, KDM3A knockdown induced the cleavage of caspase-7 and PARP in OVCAR-5/CDDP and A2780/CDDP cells (Figures 3g and h). These results clearly indicated that KDM3A knockdown itself is sufficient to induce apoptosis in platinum-resistant ovarian cancer cells. Next, we asked whether KDM3A depletion reverse the sensitivity of platinum-resistant cell lines to cisplatin treatment. To explore this, we exposed the scrambled control and KDM3A knockdown cells to cisplatin (10 and 20 μM) for 24 h and checked the expressions of apoptotic markers by western blot. Indeed, cisplatin treatment robustly induced the expression of cleaved PARP and caspase-7 in KDM3A knockdown OVCAR-5/CDDP and A2780/CDDP cells as compared with scrambled control cells (Figures 3i and j). Taken together, these results clearly indicated that KDM3A is required for the cell growth and confers chemoresistance in ovarian cancer.

KDM3A controls ovarian cancer growth and chemoresistance by modulating p53-mediated expressions of p21 and Bcl-2

To gain the molecular insight into the mechanism by which KDM3A regulates ovarian cancer growth and chemoresistance, we screened the expression of key cell cycle and apoptotic regulatory proteins in scrambled control and KDM3A knockdown OVCAR-5/CDDP cells by immunoblot. Interestingly, KDM3A knockdown induced the cyclin-dependent kinase inhibitor 1, p21<sup>Waf1/Cip1</sup>, and reduced the anti-apoptotic B-cell lymphoma 2 (Bcl-2) expressions in OVCAR-5/CDDP cells (Figure 4a). Consistently, KDM3A depletion in A2780/CDDP cells was also associated with robust induction of p21 expression with concomitant decrease in Bcl-2 levels (Figure 4b). Next, to examine whether KDM3A shRNA-induced changes in p21 and Bcl-2 expressions are regulated at transcriptional or translational level, we quantified the mRNA expression by real-time RT-PCR. In line with immunoblot, cells expressing KDM3A shRNAs showed high abundance of p21 with reduced levels of Bcl-2 mRNA (Figures 4c and d), indicating that KDM3A regulates these genes at transcriptional level. The tumor suppressor protein p53 is a well-known regulator of p21 and Bcl-2 expressions.<sup>20,21</sup> The transcriptional activity of p53 is regulated by post-translational modifications such as phosphorylation, acetylation and methylation.<sup>10,22</sup> Recent study showed that KDM3A inhibits p53 transcriptional activity by demethylating p53-K372me1 in breast cancer cells to represses apoptotic gene expression.<sup>23</sup> Therefore, we hypothesized that KDM3A may influence p21 and Bcl-2 expressions by demethylating p53-K372me1 in ovarian cancer. To check this possibility, we assessed the expression of
p53-K372me1 in scrambled control and KDM3A-depleted OVCAR-5/CDDP and A2780/CDDP cells by immunoblot. Indeed, KDM3A shRNAs consistently upregulated p53 at K372 enhancing the transcriptional activity by stabilizing the chromatin bound p53. Therefore, we quantified the p53 occupancy on p21 promoter by chromatin immunoprecipitation (ChIP) assay. Consistently, anti-p53 antibody-enriched chromatin showed increased binding of p53 protein on p21 promoter in KDM3A-depleted cells (Figure 4g). Normal immunoglobulin G (IgG)-enriched chromatin was used as negative control.

Figure 3. KDM3A depletion promotes cellular senescence and apoptosis in cisplatin-resistant ovarian cancer cells. (a and b) Senescence associated β-galactosidase staining of OVCAR-5/CDDP (a) and A2780/CDDP (b) cells expressing Scr and KDM3A shRNAs. (c and d) Quantification of β-galactosidase positive cells in OVCAR-5/CDDP (c) and A2780/CDDP (d) cells expressing Scr and KDM3A shRNAs. Each bar represents mean ± s.d. of nine random fields counted from triplicate wells. **P < 0.01, Student's t-test. (e and f) Quantification of apoptotic cells in Scr and KDM3A shRNAs expressing OVCAR-5/CDDP (e) and A2780/CDDP (f) cells. Each bar represents mean ± s.d. of triplicate samples from representative experiments. **P < 0.01, Student's t-test. (g and h) Immunoblot analysis of cleaved PARP and caspase-7 expression in scrambled control and KDM3A-depleted OVCAR-5/CDDP (g) and A2780/CDDP (h) cells. (i and j) Immunoblot analysis of cleaved PARP and caspase-7 expression in scrambled control and KDM3A knockdown OVCAR-5/CDDP (i) and A2780/CDDP (j) cells exposed to cisplatin.

KDM3A promotes ovarian cancer stemness by epigenetically activating Sox2 expression

CSC hypothesis states that subset of tumor cells with self-renewal and differentiation potential initiate tumor development and progression. These long-lived CSCs are the underlying cause of disease relapse and treatment failure in ovarian cancer because these cells are endowed with apoptosis resistance. In light of the emerging consensus on CSCs hypothesis, we attempted to investigate whether platinum resistance co-exist with stemness in ovarian cancer by utilizing A2780/CDDP cells. We conducted tumorsphere assay, which is an indicator of CSCs, using parental and cisplatin-resistant A2780 cells. Indeed, A2780/CDDP cells formed more and larger tumorsphere in suspension culture as compared to parental cells (Figure 5a). Because our findings indicated that high abundance of KDM3A coincide with cancer stemness and chemoresistance in ovarian cancer, we hypothesized that KDM3A might be a critical regulator of CSCs in ovarian cancer. To check this possibility, we determined the percentage of aldehyde dehydrogenase 1 (ALDH-1)-positive cells, a hallmark of stem cells, between A2780/CDDP cells expressing scrambled and KDM3A shRNA by fluorescence-activated cell sorting. Intriguingly, while KDM3A knockdown significantly inhibited the Sox2 and Nanog expressions in A2780/CDDP cells, Oct4 and Lin28 expressions were weakly upregulated (Figure 4d). Next we checked the Sox2 and Nanog
protein expression by western blot. Consistent with real-time RT-PCR, immunoblotting also revealed that KDM3A knockdown inhibited Sox2 expression in A2780/CDDP cells (Figure 5e). Despite the repeated attempt, we were unable to detect Nanog protein expression by immunoblot in A2780/CDDP cells. The real-time RT-PCR also revealed that the cycle threshold (Ct) value for Nanog was too high, indicating that Nanog might be weakly expressed in A2780/CDDP cells. Recent study indicated that chromatin modification through H3K9 methylation regulates Sox2 expression in lung cancer. Because KDM3A is known to activate gene expression through H3K9me2 demethylation, we checked the binding of KDM3A and the level of its substrate, H3K9me2 on Sox2 promoter by ChIP assay. Significant amount of KDM3A protein was detected on Sox2 promoter and KDM3A knockdown reduced KDM3A occupancy with concomitant increase in H3K9me2 (Figures 5f and g), indicating that KDM3A may epigenetically activates Sox2 expression to promote ovarian cancer stemness.

KDM3A is required for in vivo tumor growth and abundantly expressed in human ovarian cancer tissues

The results discussed so far clearly showed that KDM3A is crucial for tumor cell proliferation, apoptosis resistance and CSCs maintenance. Therefore, we next sought to determine the importance of KDM3A for in vivo growth of human ovarian cancer by utilizing the mouse tumor xenograft model. We subcutaneously inoculated A2780/CDDP cells expressing scrambled and KDM3A shRNAs on the right and left flanks of nude mice, respectively. Once the palpable tumors were formed, we periodically measured the tumor growth and calculated the tumor volume. Indeed, KDM3A depletion significantly inhibited the ovarian cancer growth in vivo (Figures 6a and b). To further determine whether KDM3A epigenetically activates Sox2 expression to control ovarian cancer progression, we determined KDM3A and Sox2 protein expression in human ovarian cancer tissue array containing primary and metastatic ovarian cancer and adjacent normal tissues. KDM3A and Sox2 expressions were significantly elevated in ovarian cancer than the adjacent normal tissues (Figure 5c and Table 1). Moreover, the abundance of KDM3A was positively correlated with Sox2 levels (Table 2), indicating the functional significance of KDM3A in human ovarian cancer.

**DISCUSSION**

Existence of strong association between chemoresistance and disease relapse in ovarian cancer underscores the need to identify the molecular basis of resistant phenotype to develop targeted therapy. The findings presented here demonstrated for the first time that...
time that histone demethylase, KDM3A is crucial for the ovarian cancer cells to successfully progress through the critical stages of tumor progression such as cell proliferation, maintenance of CSCs and development of chemoresistance. To control these processes, KDM3A employs two distinct mechanisms; one by demethylating histone (H3K9me2) and the other by targeting a non-histone protein, p53. Mechanistically, while activating Sox2 expression by erasing the repressive methylation (H3K9me2) mark, KDM3A modulates p21 and Bcl-2 expression possibly through p53-K372me1 demethylation. The dual mechanisms we reported here are consistent with the recent findings on breast cancer, where KDM3A induced pro-invasive genes and repressed pro-apoptotic genes by demethylating histone (H3K9me2) and non-histone protein p53, respectively.23 Even though KDM3A is mechanistically engaged in similar pathways, the target genes and cellular functions controlled by KDM3A vary between breast and ovarian cancer. Especially, KDM3A loss in ovarian cancer induces replicative senescence and cell cycle arrest but no such effects were seen in breast cancer cells. However, KDM3A renders chemoresistance in both the cancers by modulating p53 target gene expressions. In view of our finding, we attempted to analyze the patient data sets from oncomine database to identify the correlation between KDM3A expression and cisplatin resistance in ovarian cancer. Our mining indicated that no such data sets are available in the online database. However, two different studies31,32 indicated that KDM3A mRNA expression was high in ovarian serous cystadenocarcinoma than the normal ovarian epithelium (supplementary Figure 1a and b).

Hypoxic tumor microenvironment drives ovarian cancer aggressiveness by promoting the cancer stemness, cellular growth, metastasis and therapeutic resistance.33 Recently, KDM3A was identified as a potential target of hypoxia-inducible factor-1α and mediates hypoxia-induced gene expression to control tumor growth.16 Since our study indicated KDM3A as ovarian cancer oncogene and is required for tumor growth and apoptosis resistance, it is possible that KDM3A might be critical mediator of hypoxia-driven tumor aggressiveness and thus could be a potential target to inhibit hypoxia-driven ovarian cancer.

Compelling evidences suggest that tumor initiating CSCs are responsible for the development and progression of various cancers.33 Because CSCs are endowed with high metastatic and apoptosis-resistant potential, chemotherapy often failed to eliminate CSC population. Consequently, surviving CSCs initiate

Figure 5. KDM3A controls ovarian CSCs by regulating Sox2 expression. (a) Representative image of tumorsphere-formation assay indicating high abundance of CSCs in A2780/CDDP cells. (b) Flow cytometry analysis of the abundance of CSCs in A2780/CDDP cells expressing Scr and KDM3AshRNA2. (c) Tumorsphere-formation assay of Scr and KDM3AshRNA expressing A2780/CDDP cells. (d) Real-time RT-PCR of pluripotent markers expression in Scr and KDM3A shRNAs transfected A2780/CDDP cells. Each bar represents mean ± s.d. of triplicate samples from representative experiments. *P < 0.05, **P < 0.01 Student’s t-test. (e) Immunoblot analysis of Sox2 expression in A2780/CDDP cells expressing Scr and KDM3AshRNA2. (f and g) ChIP assays indicating KDM3A (f) and H3K9me2 (g) localization on Sox2 promoter in A2780/CDDP cells expressing Scr and KDM3A shRNAs. Each value is mean ± s.d. of triplicate samples from a representative experiment.
recurrant tumor growth, promote metastasis and causes treatment failure. Therefore, in addition to tumor bulk, targeting CSCs with specific inhibitors would improve the survival rate and clinical outcome in ovarian cancer patients. Hence, recent research efforts are intensified toward identifying the genetic and epigenetic changes that confers chemoresistance to CSCs. In this regard, our results indicating KDM3A as a key epigenetic factor controlling ovarian CSCs may offer a new perspective to develop an inhibitor that specifically target KDM3A to eliminate CSC population and overcome chemoresistance in ovarian cancer. Since the demethylating activity and biological functions of KDM3A resides in the catalytic subunit, it can be a potential druggable target for small molecules.

**Figure 6.** KDM3A is required for in vivo tumor growth and highly expressed in human ovarian cancer tissues. (a) Representative photograph of mice bearing the tumors formed by Scr and KDM3AshRNA2 transfected A2780/CDDP cells. (b) Tumor growth curve of subcutaneously injected Scr and KDM3AshRNA2 expressing A2780/CDDP cells in nude mice. Data are mean ± s.d., n = 5. **P < 0.01, Student’s t-test. (c) Representative images of KDM3A and Sox2 expressions in human ovarian cancer and adjacent normal tissues.

**Table 1.** KDM3A is highly expressed in human ovarian cancer tissues

|           | KDM3A | Sox2 |
|-----------|-------|------|
|           | 0     | +    | ++   | +++  | 0     | +    | ++   | +++  |
| Normal    | 11%   | 89%  | 0%   | 0%   | 33%   | 67%  | 0%   | 0%   |
|           | (1/9) | (8/9) | (0/9) | (0/9) | (3/9) | (6/9) | (0/9) | (0/9) |
| Cancer**  | 0%   | 8%   | 57%  | 35%  | 0%   | 8%   | 57%  | 35%  |
|           | (0/63)| (5/63)| (36/63)| (22/63)| (0/63)| (5/63)| (36/63)| (22/63)|

Adjacent normal ovarian tissues (Normal; n = 9) along with human ovarian cancer including metastatic tumor tissues (cancer; n = 63) were stained for KDM3A and Sox2. The intensity of the staining was scored as negative (0), weak (+), moderate (++) and strong (+++). **P < 0.01 normal vs ovarian cancer.
molecules that specifically target the rigid catalytic domain and thus can be exploited to devise a novel therapy to target-resistant ovarian cancer.

MATERIALS AND METHODS

Cell lines and generation of stable cells
Parental (OVCAR-5, SKOV3 and A2780) and cisplatin-resistant (OVCAR-5/CDDP, SKOV3/CDDP and A2780/CDDP) cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum and antibiotics (streptomycin and penicillin) at 37 °C in 5% CO₂ and 95% air. Parental and cisplatin-resistant cell lines were generously provided by Dr Oliver Dorigo, Stanford University Medical Center, Stanford, CA, USA. To stably knockdown KDM3A in platinum-resistant ovarian cancer, lentiviruses expressing human KDM3A shRNAs (TRCN0000021150 and TRCN0000021152) were harvested with lentiviral particles. After 24 h, cells were selected with antibiotics (streptomycin and penicillin) at 37 °C in 5% CO₂.

Expression of KDM3A correlates with the Sox2 levels in human ovarian cancer

| KDM3A** | 0 | + | ++ | +++ | Total |
|---------|---|---|----|-----|-------|
| Sox2    |   |   |    |     |       |
| 0       | 0 | 3 | 0  | 0   | 3     |
| +       | 1 | 7 | 3  | 0   | 11    |
| ++      | 0 | 1 | 24 | 11  | 36    |
| +++     | 0 | 2 | 9  | 11  | 22    |
| Total   | 1 | 13| 33 | 25  | 72    |

Adjacent normal ovarian tissues (n = 9) and ovarian cancer tissues (n = 63) were stained for KDM3A and Sox2. The staining score calculations were described in Table 1. Fisher’s exact test \( P = 0.000016 \).

Tumorsphere-formation assay
Cells were trypsinized and seeded (about 4000 cells/well) onto ultra-low attachment six-well culture plate containing 2.5 ml of sphere forming media (Dulbecco’s Modified Eagle Medium/F12 50:50 containing 1% supplement B, 20 ng/ml EGF and 10 ng/ml fibroblast growth factor) and cultured for 14 days. The tumospheres were photographed using the microscope.

Immunohistochemical staining of human ovarian cancer tissue
Human ovarian cancer tissue array (Cat No BC110118) containing primary and metastatic ovarian cancer and adjacent normal tissues was purchased from US Biomax, Rockville, MD, USA. The tissue array slides were deposited to UCLA pathology core for immunohistochemical (IHC) staining. The intensity of immunostaining was scored as no (0), weak (+), moderate (++) and strong (+++). The Fisher’s exact test was applied to test the differences in the staining intensity and the correlation between KDM3A and Sox2 expression in adjacent normal and ovarian cancer tissues.

Oncomine data analysis
Microarray data sets from oncomine database was analyzed for KDM3A mRNA expression between normal and ovarian cancer. The details of the methods and statistical calculations applied were described elsewhere.
Statistical analysis
The data were subjected to statistical analyses using SAS STAT version 9.1 (SAS Institute Inc., Cary, NC, USA). Independent means were compared using unpaired Student’s t-tests.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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