Chemotherapeutic Sensitivity of Testicular Germ Cell Tumors Under Hypoxic Conditions Is Negatively Regulated by SENP1-Controlled Sumoylation of OCT4

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Abstract
Testicular germ cell tumors (TGCT) generally respond well to chemotherapy, but tumors that express low levels of the transcription factor OCT4 are associated with chemoresistance and poor prognosis. Hypoxia is known to induce drug resistance in TGCTs; however, the mechanistic basis for reduced expression of OCT4 and drug resistance is unclear. Here we show that hypoxia reduces OCT4 levels and increases the resistance of embryonal carcinoma (EC) cells to cisplatin and bleomycin. Furthermore, we show that the loss of OCT4 expression under hypoxia can be triggered by sumoylation, which was regulated by SUMO1 and the SUMO1 peptidase SENP1. Under hypoxic conditions, overexpression of SUMO1gg (the active form of SUMO1) not only increased the level of sumoylated OCT4 (Su-OCT4), but also decreased the stability of OCT4 protein. In addition, overexpression of SENP1 reduced the Su-OCT4 level induced by SUMO1gg overexpression, thereby maintaining OCT4 levels and enhancing chemosensitivity. Mechanistic investigations revealed that OCT4 sumoylation occurred at K123, as overexpression of an OCT4-K123R mutant effectively reduced the level of Su-OCT4 under hypoxic conditions. Taken together, our results showed that hypoxia reduces OCT4 expression levels in ECs to increase drug resistance and that these effects could be countered to ablate the suppressive effects of hypoxia on chemosensitivity. Our findings also highlight SENP1 as a potential therapeutic target for drug resistant TGCTs. Cancer Res; 72(19): 4963–73. ©2012 AACR.

Introduction
Testicular germ cell tumors (TGCT) are the most common malignancies among human germ cell tumors and are histologically classified into seminomas and nonseminomas (1). Seminomas are generally histologically uniform and resemble a transformed state of primordial germ cells (PGC). Nonseminomas are typically heterogeneous and include teratomas, yolk sac tumors (YST), and choriocarcinomas (1). In the clinic, pluripotent seminomas and ECs of TGCTs are able to differentiate into other tumor types (e.g., teratomas, YSTs, and choriocarcinomas; 2, 3). Feldman and colleagues further proposed that differentiated TGCTs are more malignant and display higher resistance to chemotherapy (4). We therefore presumed the identification of a therapeutic target involved in the differentiation process of pluripotent TGCTs could improve the prognosis of the drug-resistant germ cell tumors.

Previous work has shown that drug resistance of TGCTs may be modulated by retinoic acid treatment (5) and/or hypoxic stresses (6). Hypoxia is also known to be an important stimulus for tumor progression and drug susceptibility. As embryos develop under hypoxic conditions, most notably during the peri-implantation period, hypoxia is also believed to be involved in modulating pluripotent cells during early embryogenesis (7). In support of this, the transcription factor OCT4, a master regulator controlling the pluripotency of embryonic stem (ES) cells (8), PGCs (9), and induced pluripotent stem (iPS) cells (10, 11), is upregulated under hypoxic conditions in ES and a wide variety of cancer cells (12–14). In TGCTs, OCT4 is upregulated in pluripotent seminomas and ECs (15) and a lack of OCT4 in ECs has been shown to increase cisplatin resistance in vitro and in vivo (16, 17). These results highlight the role of OCT4 in modulating the differentiation status and drug resistance of these tumors.
susceptibility of EC cells. We therefore hypothesized that hypoxia might increase the drug resistance of TGCT cells by modulating the level of the pluripotent transcription factor OCT4.

Sumoylation is a posttranslational modification involving covalent linkage of the small ubiquitin-like modifier (SUMO) to proteins, which regulates various cellular functions including nuclear-cytosolic transport, transcriptional regulation, apoptosis, stress responses, cell cycle progression, and protein stability (18). The sumoylation process is reversible; sumoylation and desumoylation are controlled by ubiquitin-conjugating enzyme E2I (UBE2I or UBC9) and SUMO1/sentrin specific peptidase (SENP) proteins (18). A previous report showed that the level of mouse Oct4 protein can be regulated by sumoylation; the covalent linkage of SUMO1 at lysine 118 residue (K118) increased the stability of mouse Oct4 protein under normoxic conditions (19). However, whether the sumoylation process is involved in regulating the stability of human OCT4 protein in pluripotent germ cell tumors remains to be determined.

In the current work, we show that hypoxia reduces human OCT4 protein in EC cells via sumoylation at K123. Overexpression of SENP1 in hypoxic EC cells effectively increases the level of OCT4 protein and improves drug susceptibility in vitro and in vivo. These results indicate an important role for the SENP1-mediated desumoylation process in modulating the level of OCT4 protein and the drug sensitivity of EC cells under hypoxic conditions.

Materials and Methods

Cell culture and hypoxic treatment

NCCIT (CRL-2073™), NT2 (CRL-1973™), and HEK293T cells were purchased from American Type Culture Collection. NCCIT cells were maintained in RPMI-1640 medium (Invitrogen), and NT2 and HEK293T cells were maintained in Dulbecco’s Modified Eagle’s Medium (Invitrogen). All cells were supplemented with 10% FBS at 37°C in a 5% CO2 humidified atmosphere. For hypoxic treatment, the cells were cultured at 37°C in 5% CO2/1% O2/94% N2.

In vitro sumoylation assay

The cell-free in vitro sumoylation assay was carried out using an in vitro sumoylation kit (UW8955, Enzo Life Sciences). HA-tagged OCT4 expressed in HEK293T cells was harvested by immunoprecipitation with an anti-HA-agarose antibody (A2095, Sigma-Aldrich). In brief, the harvested HA-tagged OCT4 protein was divided equally into 2 or 3 portions for sumoylation reactions [SUMO activating enzyme (E1), SUMO conjugating enzyme (E2, as UBC9), Mg-ATP] in the presence or absence of HisSUMO1 protein (UW9195, Enzo Life Sciences) and glutathione S-transferase (GST)-SENP1 (UW9760, Enzo Life Sciences). Then the reactions were incubated at 37°C for 60 minutes, washed extensively with PBS, and then stopped by adding sample buffer for SDS-PAGE separation and Western blot analysis.

Protein stability assays

HEK293T cells were transfected with either HA-OCT4-WT or HA-OCT4-K123R plasmids using a TurboFect transfection reagent (Fermentas) according to the manufacturer’s instructions. To examine the OCT4 protein stability, plasmid-transfected HEK293T cells were cultivated at 37°C for 24 hours. Then the total cells were pooled together, split equally into 6-cm plates to ensure the same cell transfection efficiency in each plate. After culture for an additional 24 hours, cycloheximide (CHX; 100 μg/mL, C4859, Sigma-Aldrich) was added to each plate, and cells were subsequently harvested at the different indicated times for SDS-PAGE separation and Western blot analysis.

Xenograft tumor models

For xenografting, 8-week-old nonobese diabetic/severe combined immunodeficient (NOD-SCID) mice were obtained from BioLasco Taiwan. NCCIT cells (10⁶ cells) infected with a lentivirus of an empty vector or HA-SENP1 were engrafted beneath the renal capsule of NOD-SCID mice according to Szot and colleagues (20). At 8 weeks after implantation, host mice were sacrificed by CO2 asphyxiation, and suterus were dissected for histologic processing. For an in vivo drug susceptibility analysis, Empty- and HA-SENP1-NCCIT cells (10⁶ cells with Matrigel) were implanted into thymic nude mice (BioLasco Taiwan) by a subcutaneous injection. The tumor volume (1/2 × length × width²) was measured every 3 or 4 days. On day 50, nude mice were injected intraorbitally with cisplatin (3 mg/kg/day) for 7 continuous days.

Statistical analysis

All experiments were repeated at least 3 times with different individual samples. Data are expressed as the mean ± SD. Statistical differences between sets of data were determined using paired 2-tailed Student t test, with P < 0.05 considered significant.

Results

Hypoxia induces drug resistance and reduces OCT4 protein in EC cells

Because hypoxia can promote the malignancy and drug resistance of EC cells (6), we initially examined whether hypoxia reduces the drug susceptibility of EC cells by altering the level of OCT4. Cisplatin and bleomycin, 2 of the most commonly used drugs for TGCT clinical chemotherapy, were used to treat NCCIT and NT2 cells under normoxia (21% O2) and hypoxia (1% O2). As shown in Fig. 1, hypoxia increased significant drug resistance to cisplatin and bleomycin in both NCCIT and NT2 cells (Fig. 1A). Moreover, hypoxia was found to decrease the level of OCT4 protein in a time-dependent manner (Fig. 1B). Quantitative analysis showed that hypoxia decreased the level of OCT4 protein by 60% in NCCIT cells and by 77% in NT2 cells after a 24-hour incubation (H24 of Fig. 1B, **, P < 0.01). However, hypoxia did not affect the mRNA level of OCT4 in EC cells (Fig. 1C). The finding that hypoxia decreased OCT4 protein level in NCCIT cells was confirmed using immunocytochemical staining combined with confocal microscopy (Supplementary Fig. S1). These observations suggest that hypoxia may regulate the stability of OCT4 protein via a posttranslational modification.
Hypoxia regulates the stability of the OCT4 protein in EC cells through sumoylation

Because sumoylation can modulate the stability of Oct4 protein in mouse pluripotent cells (19), we examined whether overexpression of EGFP-SUMO1gg (an active form of SUMO1) could lead to sumoylation and destabilization of the OCT4 protein in EC cells.

Overexpression of EGFP-SUMO1gg resulted in a dose-dependent decrease in the level of the OCT4 protein in NCCIT cells (Figs. 2A and Supplementary Fig. S2). In the presence of CHX, hypoxia further decreased the level of the OCT4 protein in EGFP-SUMO1gg-NCCIT cells, compared with CHX treatment under normoxic conditions (Fig. 2B). These results suggest that hypoxia-mediated sumoylation affects the stability of the OCT4 protein in NCCIT cells.

The finding that sumoylation of the OCT4 protein under hypoxia affects its stability was further supported by immunofluorescent staining experiments. As shown in Fig. 2C, immunofluorescent staining of NCCIT cells in the presence of 10 μmol/L MG132, a proteasome inhibitor, showed stronger colocalization of OCT4 protein (red) and EGFP-SUMO1gg under hypoxic conditions compared with either SUMO1aa overexpression or normoxic conditions (colocalization is indicated by yellow, +MG132 vs. −MG132 panel). Western blotting also showed that hypoxia enhanced the generation of high-molecular-weight sumoylated-OCT4 (Su-OCT4) in NCCIT cells expressing EGFP-SUMO1gg (Fig. 2D, lanes 5 and 6 vs. lane 4, indicated by an arrowhead), but not in NCCIT cells expressing EGFP-SUMO1aa (Fig. 2D, lanes 1–3). The unsumoylated OCT4 is shown for both short- and long-exposure times (Fig. 2D, indicated by arrows). These results further support OCT4 sumoylation under hypoxia reducing the stability of the OCT4 protein in NCCIT cells.

Sumoylation of OCT4 protein occurs at K123

A previous study showed that sumoylation at K118 regulates the stability of the Oct4 protein in mice (19). However, the
The sumoylation site of the human OCT4 protein is unknown. Using the sumoylation motif of mouse Oct4 (yKxD/E) as a reference, K123 and K222 were identified as potential sumoylation sites in the human OCT4 (Fig. 3A). To examine sumoylation at these sites in the human OCT4 protein, an HA-tagged wild-type OCT4 expression plasmid (HA-OCT4-WT) and 3 HA-tagged mutant OCT4 expression plasmids were constructed. The K123 amino acid residue was mutated to R in the peptide produced from the HA-OCT4-K123R mutant expression plasmid, and the HA-OCT4-K222R construct produced a peptide that contained the same substitution at Position 222. The double-mutant expression plasmid, HA-OCT4-2KR, produced a peptide with both K to R substitutions. The wild type and mutant expression plasmids were transfected into HEK293T cells, and the HA-OCT4 proteins were pulled down by anti-HA agarose beads for cell-free in vitro sumoylation assays. As shown in Fig. 3B, the in vitro sumoylation assay showed a unique sumoylation site at Position 123 of the HA-OCT4 protein. The high-molecular-weight sumoylated forms of the HA-OCT4-WT and HA-OCT4-K222R proteins are shown in lanes 2 and 7, respectively, and GST-SENP1 effectively reduced the levels of the Su-OCT4 protein (lane 3 vs. lane 2).

To examine the direct interaction of endogenous OCT4 and SUMO1 in EC cells, pull-down assays with antibodies against endogenous OCT4 were conducted to isolate the OCT4 protein from NCCIT and NT2 cells. Western blotting with anti-OCT4, anti-SUMO1, and anti-rabbit immunoglobulin G antibodies showed a high-molecular-weight Su-OCT4 (Fig. 3C). To further confirm this result, plasmids of HA-OCT4-WT, -K123R, -K222R, or -2KR were cotransfected with FLAG-SUMO1gg and UBC9 into HEK293T cells for in vivo coimmunoprecipitation assays. As shown in Fig. 3D, the Su-OCT4 was detected in WT- and K222R-HEK293T cells (lanes 2 and 7), and coexpression of SENP1 significantly reduced the level of Su-OCT4 (lane 3 vs. lane 2). Sumoylation of OCT4 was further
confirmed by cotransfection of HEK293T cells with HA-OCT4 (WT, K123R, K222R, or 2KR) and the EGFP-SUMO1aa or EGFP-SUMO1gg plasmid. As shown in Fig. 3E, EGFP-SUMO1gg significantly increased the sumoylation of the wild-type OCT4 protein in HEK293T cells (lane 4 vs. lane 3), and the level of sumoylated HA-OCT4-WT was significantly reduced by coexpression of HA-SENP1 (lane 5 vs. lane 4). High-molecular-weight Su-OCT4 was not detected in cells cotransfected with HA-OCT4-K123R (lane 7 vs. lane 4) and HA-OCT4-2KR mutant constructs (lane 11 vs. lane 4), respectively. These results show that sumoylation of the human OCT4 protein occurs at K123.

Because hypoxia significantly decreased the level of OCT4 protein in EC cells (Fig. 1), we examined whether hypoxia regulates OCT4 protein stability through sumoylation. For this purpose, HEK293T cells were cotransfected with HA-OCT4 (WT, K123R, K222R, or 2KR) and the EGFP-SUMO1aa or EGFP-SUMO1gg plasmids and were incubated under normoxic and hypoxic conditions (left). The expression ratios of Su-OCT4: OCT4 for cell separately expressing the HA-OCT4-WT and the HA-OCT4-K222R proteins were observed (right). G, the half-life of the OCT4 protein in the presence of cycloheximide (100 μg/mL) in HEK293T cells transfected with the HA-OCT4-WT or the HA-OCT4-K123R plasmid under normoxic or hypoxic conditions (top). Decreased levels of OCT4 proteins in cells were quantified (bottom). Arrow, unsumoylated OCT4 (OCT4); arrowhead, sumoylated OCT4 (Su-OCT4); asterisk, nonspecific signals; IB, immunoblot; IP, immunoprecipitation; *P < 0.05.
confirm that the sumoylation of human OCT4 protein occurs at K123. We also found that hypoxia significantly increased the Su-OCT4 level in cells that were cotransfected with HA-OCT4-WT (lane 2 vs. lane 1) or HA-OCT4-222R (lane 8 vs. lane 7). The unsumoylated OCT4 protein level is shown in both long and short exposures (indicated by arrows). Quantitative analysis showed that the ratio of Su-OCT4/OCT4 in HA-OCT4-WT- and HA-OCT4-222R-HNK293T cells was significantly increased under hypoxia (Fig. 3F, right panel).

To further examine whether hypoxia decreased the stability of OCT4, HEK293T cells were transfected with HA-OCT4-WT and HA-OCT4-K123R expression plasmids before treatment with CHX, and cells were harvested at various time points. As shown in Fig. 3G, hypoxia decreased the half-life of OCT4 in the presence of CHX, compared with similar normoxic conditions (WT panel), and the wild-type OCT4 protein displayed a longer half-life than did the mutant HA-OCT4-K123R protein under hypoxic conditions. These results collectively show that hypoxia enhances sumoylation of the human OCT4 protein at K123, which leads to a reduction in the stability of the human OCT4 protein.

**SENP1 suppresses OCT4 sumoylation and increases OCT4 stability under hypoxic conditions**

SENP1 is a negative regulator of protein sumoylation. Initially, we found that the level of SENP1 transcription was lower in NCCIT/NT2 cells compared with human ES cells (Supplementary Fig. S3). We further found that overexpression of SENP1 in NCCIT cells suppressed hypoxia-induced reduction of the OCT4 protein. NCCIT cells were infected with a lentivirus carrying an empty vector (control vector) or HA-SENP1-IRES-tRFP (HA-SENP1). As shown in Fig. 4A, immunofluorescent staining showed that higher expression of SENP1 (indicated by tRFP, red fluorescence) correlated with the more intense OCT4 staining (indicated by fluorescein isothiocyanate, green fluorescence). Western blotting further confirmed that SENP1 can suppress hypoxia-induced reduction of the OCT4 protein in NCCIT cells. Quantitative analysis showed that overexpression of HA-SENP1 significantly increased the OCT4 protein level in hypoxic conditions (Fig. 4B; ***, P < 0.05, n = 3). The increased stability of the endogenous OCT4 protein resulting from coexpression of SENP1 was further confirmed by CHX treatment. The HA-SENP1-NCCIT cells contained a higher level of the OCT4 protein after the 12 hours incubation under normoxic conditions, compared with the vector control group (Supplementary Fig. S4).

In contrast, knockdown of UBC9, a SUMO-conjugating enzyme, in cells by shRNAs blocked hypoxia-induced reduction of the OCT4 protein (Fig. 4C; H24, shLuc vs. shUBC9). These results support the assertion that sumoylation of the OCT4 protein under hypoxia affects its stability in NCCIT cells.

Regulation of the OCT4 protein stability by SENP1 was further examined using tumor-bearing mice models. Control NCCIT cells (infected with an empty lentiviral vector) and SENP1-overexpressing NCCIT cells (infected with lenti-HA-SENP1) were transplanted into renal capsules of NOD-SCID mice. As shown in Fig. 4D, tRFP staining marked grafted tumor tissues (Fig. 4D, tRFP panel). Compared with the empty vector group, NCCIT cells that expressed HA-SENP1 had more intense OCT4 staining (Fig. 4D; HA-SENP1 panel vs. Empty vector panel). Lower cytokeratin expression was correlated with intense OCT4 staining and was observed in NCCIT cells that expressed HA-SENP1 compared with empty vector control cells. Quantitative analysis confirmed that there were more OCT4-positive cells in the tumor generated from transplantation of SENP1-overexpressed NCCIT cells than in the tumor generated from transplantation of control NCCIT cells. The finding that OCT4 staining was negatively correlated with cytokeratin staining shows that SENP1 suppresses the differentiation of NCCIT cells in vivo via maintaining the level of the OCT4 protein (Fig. 4E).

**SENP1 increases the drug sensitivity of EC cells in hypoxic conditions**

Low levels of OCT4 are known to be associated with higher drug resistance in ECs (16, 17). Because SENP1 can increase OCT4 protein stability in hypoxic conditions (Fig. 4), we examined the effect of SENP1 on the drug sensitivity of EC cells. As shown in Fig. 5, higher drug sensitivity was observed in SENP1-overexpressing NCCIT cells compared with control NCCIT cells (Fig. 5A; *, P < 0.05; ***, P < 0.01). The IC50 value of HA-SENP1-overexpressing NCCIT cells in hypoxic conditions (21.63 ± 0.12 μmol/L for cisplatin and 7.33 ± 0.14 μmol/L for bleomycin) was greatly decreased to a level similar to that of control or HA-SENP1-overexpressing NCCIT cells in normoxic conditions (16.9 ± 0.83 μmol/L for cisplatin and 3.72 ± 0.52 μmol/L for bleomycin; Supplementary Table S3). There was no significant difference in cell viability between control and HA-SENP1-overexpressing NCCIT cells in either normoxic or hypoxic conditions (Supplementary Fig. S5). Xenograft experiments using nude mice (n = 35) were conducted to examine the effect of SENP1 on the drug susceptibility of EC cells in vivo. In these experiments, Control NCCIT cells (infected with empty lentiviral vector) and SENP1-overexpressing NCCIT cells (infected with lenti-HA-SENP1) were injected into nude mice, and the tumor volume was determined 50 days postinjection (Fig. 5B). The tumor-bearing mice were then treated with cisplatin for 7 continuous days (3 mg/kg/day, via intraperitoneal injection) and tumor size was measured on days 1, 4, and 7. When compared with the day 1 control group, cisplatin significantly decreased the size of the tumor in the HA-SENP1 group (35% vs. 73% decrease; Fig. 5C). Hematoxylin and eosin (H&E) staining showed that the tumors in the HA-SENP1 group were poorly differentiated compared with the control group tumors (Fig. 5D). The positive hypoxia-inducible factor-1α (HIF-1α) expression indicates the hypoxic microenvironment in tumor regions (Fig. 5D). Importantly, fewer tRFP-labeled and p21-expressing cells were observed in the HA-SENP1 groups, suggesting that SENP1-overexpressing NCCIT cells were sensitive to cisplatin treatment (Fig. 5D). Positive staining of cleaved caspase-3 in the HA-SENP1 groups (Fig. 5D) further supported the higher cisplatin susceptibility of SENP1-overexpressing NCCIT cells. Quantitative analysis confirmed the observation and showed that SENP1 increased the susceptibility of NCCIT cells to cisplatin treatment in vivo (Fig. 5E).
SENP1 enhances the drug sensitivity of EC cells by maintaining the level of the OCT4 protein

To examine whether the SENP1 protein increases the drug sensitivity of EC cells by maintaining the OCT4 protein stability, we knocked down the expression of the endogenous OCT4 protein in NCCIT cells in which HA-SENP1 was simultaneously overexpressed in the presence of cisplatin or bleomycin. Knockdown of OCT4 and the overexpression of HA-SENP1 in NCCIT cells are shown in Fig. 6A. Under both normoxic and hypoxic conditions, OCT4 silencing significantly increased IC_{50} to cisplatin and bleomycin in both the HA-SENP1-NCCIT cells (Fig. 6B, lanes 7–12) and the NCCIT control cells (Fig. 6B, lanes 1–6), clearly demonstrating an inverse relationship between the level of endogenous OCT4 protein and the IC_{50} value (Fig. 6B). These results show that SENP1 regulates susceptibility to cisplatin and bleomycin through its effects on the level of OCT4 protein in EC cells.

Discussion

OCT4 is a key transcription factor involved in regulating the self-renewal and pluripotency of ES cells and PGCs (8, 9). Recent work has also shown that OCT4 has a key role in
reprogramming somatic cells to a pluripotent stage (11). Most TGCTs express OCT4, suggesting that it plays a critical role in germ cell neoplasia and that it may be useful as a marker for preinvasive and invasive TGCTs (21, 22). A portion of TGCTs display resistance to chemotherapy, and this chemoresistance is associated with several genetic elements (23) including the loss of OCT4 expression. Recent evidence has showed that loss of OCT4 in EC cells increases resistance to cisplatin treatment (16, 17). However, the mechanism underlying the loss of OCT4 in EC cells is less clear. OCT4 is known to be regulated by multiple processes, including epigenetic methylation (24), posttranslational sumoylation (19), and hypoxic oxygen tension (25). Hypoxia is known to affect the level of OCT4 expression in ES cells (12), PGCs (25), iPSC cells (26), and a wide variety of cancer stem cells (CSC; 13, 14). For example, hypoxia can increase the level of OCT4 expression in ES cells through stabilizing the hypoxia-inducible protein HIF-2α. It has been shown that HIF-2α binds to the hypoxia response element of the OCT4 promoter, and then activates OCT4 expression (25). We also found that hypoxia increased the HIF-2α level in ES cells (Supplementary Fig. S6A), but not in EC cells (Supplementary Fig. S6B). This result may explain why
hypoxia increased the OCT4 expression in ES cells, but decreased the OCT4 protein level in EC cells (Supplementary Fig. S6). The discrepancy of hypoxia effect on the levels of HIF-2α and OCT4 in ES and EC cells may be because of the different expression levels of SENP1 in these cells. SENP1 has been showed to be a regulator of HIF-2α protein stability (27). Compared with ES cells, the SENP1 level in EC cells is extremely low (around 20% of the ES cells; Supplementary Fig. S3). This low expression of SENP1 may explain the low HIF-2α and OCT4 protein levels in EC cells under hypoxia (Supplementary Figs. S3 and S6).

SENP1 can cause desumoylation by removing the SUMO group from target proteins. The current work showed that SENP1 can reduce the sumoylation of OCT4 protein induced by hypoxia in EC cells, leading to increased OCT4 protein stability and enhancement of drug sensitivity. Hypoxia is known to increase protein sumoylation-associated gene expression, including SUMO1 and RSUME (a small RWD-containing protein that is a sumoylation enhancer; 28, 29). In a SENP1−/− mice model, hypoxia induces sumoylation of HIF-1α and drives its ubiquitination and protein degradation (30). Removing a SUMO1 group from the nuclear HIF-1α by SENP1 activates HIF-1α and HIF-1β complex formation, thus driving the transcriptional activation of EPO, VEGF, and Glut-1 (30). Moreover, a sumoylation modification at the specific K118 residue increases mouse Oct4 protein stability under normoxic conditions (19). In our results, hypoxia downregulated SENP1 protein level (Supplementary Fig. S7A) and upregulated the RSUME mRNA level (a sumoylation enhancer; Supplementary Fig. S7B), and decreased human OCT4 stability in EC cells through sumoylation modification at K123 (Figs. 2 and 3). Overexpression of SENP1 in EC cells removed SUMO1 from OCT4 (Fig. 3B, D–F), and increased OCT4 protein stability and drug susceptibility (Figs. 4 and 5).

Loss of OCT4 in EC cells has been shown to increase resistance to cisplatin treatment (23), but the mechanism behind this remains to be determined. In several types of CSCs, hypoxia is known to increase the OCT4 protein level, thereby enhancing drug resistance (31, 32). For example, signaling of AKT and ABCG2 is known to mediate OCT4-induced drug resistance in hepatocellular carcinomas (33). However, OCT4 seems to play an opposite role in EC cells. Reduction of OCT4 via hypoxia or retinoic acid treatment in EC cells has been shown to be associated with increase in drug resistance (5, 6). The mechanisms that lead to increase in drug resistance in differentiated EC cells have been proposed to involve epigenetic remodeling and p21 regulation (34, 35). In addition to the role of epigenetic remodeling, our current work shows that overexpression of SENP1 in EC cells under hypoxia significantly restores the level of OCT4 protein and increases drug susceptibility both in vitro and in vivo (Figs. 4 and 5). The role of SENP1 in enhancing the drug susceptibility of EC cells is supported by the lower expression of cytoplasmic p21 and the higher level of cleaved caspase-3 found in the HA-SENP1-xenograft tumors (Fig. 5D and E). These results are consistent with previous work showing that OCT4 negatively
regulates p21, which is highly expressed in chemoresistant germ cell tumors and protected against cisplatin-induced apoptosis (35).

In conclusion, the current work shows that hypoxia decreases the level of OCT4 protein in EC cells via sumoylation at K123 (Fig. 7). Overexpression of SENP1 in EC cells under hypoxia can effectively restore the level of OCT4 protein and improve drug sensitivity in vitro and in vivo. These findings suggest that SENP1 may be a promising therapeutic target for drug-resistant TGCTs.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: Y.-C. Wu, T.-Y. Ling, S.-H. Lu, H.-N. Ho, C.-N. Shen, Y.-H. Huang
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