DDB2 Suppresses Tumorigenicity by Limiting the Cancer Stem Cell Population in Ovarian Cancer

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Abstract

Ovarian cancer is an extremely aggressive disease associated with a high percentage of tumor recurrence and chemotherapy resistance. Understanding the underlying mechanism of tumor relapse is crucial for effective therapy of ovarian cancer. DNA damage binding protein 2 (DDB2) is a DNA repair factor mainly involved in nucleotide excision repair. Here a novel role was identified for DDB2 in the tumorigenesis of ovarian cancer cells and the prognosis of ovarian cancer patients. Overexpressing DDB2 in human ovarian cancer cells suppressed its capability to recapitulate tumors in athymic nude mice. Mechanistic investigation demonstrated that DDB2 is able to reduce the cancer stem cell (CSC) population characterized with high ALDH activity in ovarian cancer cells, probably through disrupting the self-renewal capacity of CSCs. Low DDB2 expression correlates with poor outcomes among ovarian cancer patients, as revealed from the analysis of publicly available gene expression array datasets. Given the finding that DDB2 protein expression is low in ovarian tumor cells, enhancement of DDB2 expression is a promising strategy to eradicate CSCs and would help to halt ovarian cancer relapse.

Implications: DDB2 status has prognostic potential and elevating its expression eradicates CSCs and could reduce ovarian cancer relapse.
Introduction

Epithelial ovarian cancer (EOC) is the fifth leading cause of cancer-related deaths in women in the United States and the leading cause of gynecologic cancer deaths. Most of the tumors are initially responsive to platinum (Pt)-based chemotherapy and the patients enter into clinical remission after initial treatment. However, recurrence occurs in more than 70% of patients despite treatment (1). The high relapse rate in ovarian cancer results in greater mortality and are estimated to account for 5% of all deaths by cancer in women for 2013 (2). Therefore, reducing ovarian cancer relapse is especially important to prolonging progression free survival and decreasing the mortality in ovarian cancer patients.

Over the last several years, it has been increasingly evident that a small population of cancer cells, referred to as “cancer stem cells (CSCs)”, is the most important trigger of tumor progression (3, 4). The CSC theory suggests that tumor cells are organized hierarchically with a small self-renewing population of stem cells generating a large population of proliferative cells to maintain the tumors. These CSCs have been identified in a variety of solid tumors including ovarian cancers (5-8). Each type of CSC has a distinctive pattern of surface markers (i.e., CD44, CD133, CD117) and non-surface markers [i.e., aldehyde dehydrogenase (ALDH) activity] that can be targeted for CSC isolation (9). In addition, CSCs can also be isolated by detection of side-population phenotypes with Hoechst 33342 dye efflux technique (10) and their ability to grow as floating spheres in serum-free medium (11). Ovarian CSCs have been successfully isolated based on the expression of distinctive cell surface markers CD44, CD117, MyD88, and CD133 (5, 12, 13), as well as the activity of ALDH (13). All isolated ovarian CSCs fulfill all currently accepted criteria of the existence of a subpopulation of tumor-initiating cells.

CSCs possess several key properties including (i) self-renewal, (ii) multipotent differentiation into nontumorigenic cells, (iii) resistance to toxic xenobiotics, and (iv) the ability to induce tumors when transplanted into immunodeficient mice (14). A number of reports support the presence of rare CSCs that are resistant to chemotherapy and radiotherapy. These resistant CSCs are believed to be the main source
of tumor relapse (15). Thus, there is an urgent need for detailed characterization of these CSCs in order to devise new treatment modalities.

DDB2 is a 48-kDa protein originally identified as a component of the damage-specific DNA-binding heterodimeric complex DDB (16). DDB2 is able to bind UV-damaged DNA and serves as the initial damage recognition factor during nucleotide excision repair (NER) (17). The low expression of DDB2 in cisplatin-resistant ovarian cancer cell lines (18) and high-grade colon cancer (19) and skin cancer (20) indicates a link between DDB2 expression and tumor progression. Recently new functions of DDB2 beyond its role in DNA repair have been identified, e.g., inhibiting cellular apoptosis through downregulation of Bcl-2 (18, 21), and p21 (22), suppressing colon tumor metastasis through blocking epithelial-mesenchymal transition (EMT) (19), and limiting the motility and invasiveness of invasive human breast tumor cells by regulating NFκB activity (23), as well as mediating premature senescence (24). In this study, we reveal a novel role of DDB2 in the inhibition of tumorigenesis. DDB2 overexpression resulted in a reduction of the CSC population associated with repression of the tumorigenicity of ovarian cancer cells, whereas DDB2 knockdown resulted in an expansion of the CSC population.
Material and Methods

Cell culture

Human ovarian cancer cell line A2780 and its derived cisplatin-resistant cell line CP70 (25) were kindly provided by Dr. Paul Modrich (Duke University). Ovarian cancer cell line 2008 and its resistant cell line 2008C13 (26) were kindly provided by Dr. Francois X. Claret (University of Texas - M.D. Anderson Cancer Center). The A2780-derivative and 2008-derivative cisplatin-resistant cell lines were produced by intermittent, incremental exposure of the sensitive parental cell line to various concentrations of cisplatin. SKOV3 ovarian cancer cell line was kindly provided by Dr. Thomas C. Hamilton (Fox Chase Cancer Center). CP70 cells with overexpression of DDB2 (CP70-DDB2) were established in our lab (18). All cell lines were authenticated by DNA (STR) profiling, and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 \( \mu \)g/ml streptomycin and 100 units/ml penicillin. Cells were grown at 37°C in humidified atmosphere of 5% CO\(_2\) in air.

Tissue Microarray and Immunohistochemistry

Confirmed, formalin-fixed, paraffin-embedded human ovarian normal tissue and epithelial tumors were obtained from the Department of Pathology, The Ohio State University. Cores were obtained from the most viable/non-necrosis areas of the tissue. Each sample had 2 independent cores. A tissue microarray (TMA) was constructed from 16 normal tissues and 43 ovarian cancer patients (Grade 1: 4; Grade 2: 14; Grade 3: 25). TMA sections were subjected to deparaffinization and rehydration. The endogenous peroxidase was quenched by 3% (v/v) hydrogen peroxide. The epitope retrieval was performed using Dako TRS solution (Dako, Carpinteria CA) for 25 minutes at 96°C in a vegetable steamer. Primary mouse antibody against DDB2 (ab51017, 1:10, abcam, Cambridge MA) was incubated for 1 h at room temperature, and detected using Mach 3 Mouse HRP-polymer kit (Biocare Medical, Concord CA) and diaminobenzidine teterhydroxychloride (Dako). Tissues were counterstained with
Richard Allen hematoxylin. Intensity of staining was blind scored from 1 (no staining) to 4 (highest intensity of staining).

**Plasmids and gene transfection**

pReceiver-Lv105-DDB2 (GeneCopoeia, Rockville, MD) and pcDNA3.1-His-DDB2 plasmids were introduced into cells by using either lentivirus infection or electroporation with NEPA-21 Electroporator (Nepa Gene Co., Ltd, Chiba, Japan). DDB2 expression lentiviruses were generated as described before (21). To establish shDDB2 stably transfected cell lines, MISSION shDDB2 (TRCN0000083993) plasmids (Sigma) were transfected into 2008 cells using electroporation. The transfected cells were selected in the medium containing 2 µg/ml Puromycin, and the transfectant lines with stable DDB2 downregulation were confirmed by Western blotting.

**Immunoblotting**

Whole cell lysates were prepared by boiling cell pellets for 10 min in SDS lysis buffer [2% SDS, 10% Glycerol, 62 mM Tris-HCl, pH 6.8 and a complete mini-protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN)]. After protein quantification with Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Hercules, CA), equal amounts of proteins were loaded, separated on a polyacrylamide gel, and transferred to a nitrocellulose membrane. Protein bands were immuno-detected with appropriate antibodies, e.g., goat anti-DDB2 (R&D Systems, Minneapolis, MN), rabbit anti-Nanog (Cell Signaling Technology, Danvers, MA), mouse anti-Tubulin (Millipore, Billerica, MA), and mouse-anti-IκBα (Cell Signaling Technology, Danvers, MA).

**Semisolid colony forming assay**

Cells were trypsinized and counted. 1,000 cells were mixed with semisolid media (MethoCult H4100, STEMCELL Technologies Inc, Vancouver, Canada) containing serum-free DMEM/F12 (Life Technologies, Grand Island, NY) supplemented with 20% KnockOut Serum Replacement (Life
Technologies), 20 ng/ml EGF (Life Technologies), 10 ng/ml bFGF (Life Technologies), 100 μg/ml streptomycin and 100 units/ml penicillin (Life Technologies), and seeded in 6-well Ultra-Low Attachment plates (Corning, Lowell, MA). The number of larger (more than 50 cells), symmetric and prototypical colonies was counted after 6 days.

**Flow cytometry analysis and cell sorting**

Anti-CD117-PE and anti-CD44-FITC (BD Pharmingen, San Diego, CA) were used for flow cytometric analysis and cell sorting. Detection of ALDH activity was conducted using the ALDEFLUOR assay (STEMCELL Technologies) according to the manufacturer’s instruction. For each sample, one half of cells was treated with 50 mmol/L diethylaminobenzaldehyde (DEAB) to define negative gates. Flow cytometric analysis and sorting were performed on a BD FACS Aria III at The Ohio State University Analytical Cytometry Shared Resource. CP70-ALDH⁺, 2008-CD44⁺CD117⁺ and 2008C13-CD44⁺CD117⁺ cells were maintained in Ultra-Low attachment plates in KnockOut DMEM/F12 medium supplemented with 20% KnockOut Serum Replacement, 20 ng/ml EGF, 10 ng/ml bFGF, 100 μg/ml streptomycin and 100 units/ml penicillin. Cells were grown at 37°C in humidified atmosphere of 5% CO₂ in air.

**Isolation of putative CSCs based on sphere formation**

CSCs are able to form colonies from a single cell more efficiently than their progeny (27) and to grow as spheres in non-adherent culture conditions (28). We seeded SKOV3 cells in Ultra-Low attachment 96-well plate by serial dilution, and cultured them in 100 μl KnockOut DMEM/F12 medium supplemented with 20% KnockOut Serum Replacement, 20 ng/ml EGF and 10 ng/ml bFGF. 10 μl of fresh medium was added every 2 days. After 1 month, wells that contain just a single colony were marked. These SKOV3-spheroids were then subcultured from the wells into larger vessels for further growing and defined as putative CSCs.
Sphere-forming assay

1,000 of CSCs were plated in triplicate in Ultra-Low attachment plates in serum-free DMEM/F12 medium supplemented with serum replacement, EGF and bFGF. Sphere formation was assessed 2 weeks after cell seeding. Spheres are defined as floating non-adherent multicellular cell aggregates.

Quantitative RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen), and the first strand cDNA was generated by the Reverse Transcription System (Promega, Madison, WI) in a 20 μl reaction containing 1 μg of total RNA. A 0.5 μl aliquot of cDNA was amplified by Fast SYBR Green PCR Master Mix (Life Technologies) in each 20 μl reaction with the following primers: DDB2- forward, 5’- CTCCCTCAATGGAGGGAACAA -3’, reverse, 5’- GTGACCACCATTCCGGCTACT -3’; Nanog- forward, 5’- GTCCCAAAGGCAAACAACC -3’, reverse, 5’- TTGACCGGGACCTTGTCTTC -3’; GAPDH-forward, 5’- GAAGGTGAAGGTCGGAGT -3’, reverse, 5’- GAAGATGGTGATGGGATTTC -3’. PCR reactions were run on the ABI 7900 Fast Real-Time PCR system in the OSUCCC Nucleic Acid Core Facility.

Xenograft tumor growth

Athymic NCr-nu/nu mice and NOD/SCID mice (6-8 weeks, female, 20 to 25 g body weight) were obtained from National Cancer Institute (Frederick, MD). Animals were maintained in accordance with institutional policies, and all studies were performed with approval of the Institutional Animal Care and Use Committee of The Ohio State University. To assess the tumorigenicity of DDB2-overexpressing CP70 cells, 5 × 10⁶ cells were resuspended (1:1) in PBS:Matrigel (BD Biosciences, San Jose, CA) and injected subcutaneously into the flank of nude mice. To evaluate the effect of DDB2 on the tumorigenicity of SKOV3-spheroids, pcDNA3.1-His-DDB2 plasmids or empty vectors were co-transfected with pcDNA3.1-GFP plasmids into SKOV3-spheroids by electroporation. Cells were cultured for 2 days, and viable cells with GFP expression were sorted by fluorescence-activated cell sorting.
(FACS). $1 \times 10^4$ of these cells were mixed (1:1) with Matrigel and injected subcutaneously into the axillae of NOD/SCID mice. Tumor growth was measured using calipers, and volumes were calculated based on the formula $V=(a \times b^2)/2$, in which $a$ is the longest and $b$ is the shortest diameter of the tumor. Tumor growth curves were compared using a Student’s $t$ test.

**Statistical analysis**

Student’s $t$ test and Non-Parametrics Mann-Whitney test were performed for data analysis by using Minitab software. Log-rank analysis was used to determine statistical significance of Kaplan-Meier survival curve. For all statistical methods, $P < 0.05$ was considered statistically significant.
Results

DDB2 is downregulated in ovarian cancers

It has been reported that DDB2 protein expression is downregulated in human colon cancer and skin cancer demonstrated by immunohistochemical (IHC) staining (19, 20). To determine the DDB2 protein expression level in primary ovarian carcinoma, we constructed TMA and analyzed DDB2 expression using IHC with an antibody specific for DDB2. IHC staining of the samples showed that normal ovary epithelial cells exhibit strong DDB2 staining, while ovarian carcinoma cells in most of tumor tissue samples display low DDB2 expression (Fig. 1A). Statistical analysis revealed a significant decrease of DDB2 expression in ovarian carcinomas regardless the grade (Fig. 1B), indicating a strong correlation between the loss of DDB2 expression and the ovarian carcinoma.

DDB2 represses tumorigenicity of ovarian cancer cells in vivo and in vitro

To further investigate the role of DDB2 in the tumorigenicity of ovarian cancer cells, we overexpressed DDB2 in cisplatin-resistant ovarian cancer cell line CP70, which expresses low level of endogenous DDB2 (18), and established two stable cell lines with DDB2 overexpression (Fig. 2A, left panel). We also transfected DDB2 shRNA into another ovarian cancer cell line 2008 and selected two stable DDB2-knockdown cell lines (Fig. 2A, right panel). We then assessed the anchorage-independent growth of these cells in vitro by culturing them in semisolid serum-free medium. As shown in Fig. 2B-E, DDB2 overexpression significantly inhibited anchorage-independent growth of CP70 cells, while DDB2 knockdown promoted anchorage-independent growth of 2008 cells.

We next sought to determine the effects of DDB2 overexpression on the tumorigenicity of CP70 cells in vivo in immunocompromised mice. CP70-Vector cells and two clones of CP70-DDB2 cells were injected separately into nude mice subcutaneously. Tumors were formed in all mice injected with CP70-Vector cells within 1 month, while tumors could only be found in 3 out of 9 mice injected with two different CP70 cell lines stably overexpressing DDB2 up to 3 months. The tumor growth curves and the
final tumor sizes also indicate that DDB2 overexpression suppresses the growth of implanted human ovarian cancer cells (Fig. 2F,G). Taken together, these *in vitro* and *in vivo* data indicate that DDB2 is able to suppress tumorigenicity of ovarian cancer cells.

**DDB2 overexpression reduces the CSC subpopulation in ovarian cancer cells**

It has recently been reported that DDB2 suppresses the tumorigenicity and invasiveness of colon cancer cells through suppressing EMT (19). We then analyzed the EMT markers in CP70 cells with or without overexpression of DDB2. We were unable to find any effect of DDB2 overexpression on the cellular levels of epithelial marker E-Cadherin and mesenchymal marker Vimentin (Supplementary Fig. S1A). In addition, we failed to see any change in morphology of the cells with DDB2 overexpression (Supplementary Fig. S1B). Similarly, we did not find any change of EMT in various clones of ovarian cancer cell line 2008 with stable DDB2 knockdown (Supplementary Fig. S1C). These results indicate that DDB2 may not suppress EMT of ovarian cancer cells.

CSCs are thought to contribute to the initiation of the tumor (15), we thus hypothesized that DDB2 may regulate the amount of CSC subpopulation in cancer cells. Aldehyde dehydrogenase (ALDH) has been proposed to be a marker of both normal and cancer stem cells (29) and has been used to identify CSCs from ovarian cancers (30). We thus analyzed the abundance of putative CSC subpopulations characterized with ALDH$^+$ in CP70 and DDB2-overexpressing CP70 cells using FACS. It is clear that two DDB2-overexpressing CP70 cell lines exhibit reduced ALDH$^+$ subpopulation, in comparison with CP70-Vector cells (Fig. 3A,B, Supplementary Fig. S2A,B). Our immunoblotting analysis also revealed diminished ALDH1A1 expression in both DDB2-overexpressing CP70 cell lines (Supplementary Fig. S3). In contrast, downregulation of DDB2 expression in A2780 cells increased the percentage of ALDH$^+$ cells (Fig. 3C-E), further supporting that DDB2 is able to decrease the abundance of ALDH$^+$ cells in ovarian cancer cells. In addition, we also analyzed ALDH$^+$ cells in DDB2-knockdown 2008 cells and demonstrated the similar result (Supplementary Fig. S4A,B). Besides ALDH activity, CD44$^+$CD117$^+$ was also used as a phenotypic marker of ovarian CSCs (5). We then sought to determine whether DDB2
expression also affects the percentage of CD44+CD117+ fraction. Indeed, we found a decrease in CD44+CD117+ population in CP70 cells with DDB2 overexpression, and an increase in CD44+CD117+ population in 2008 cells with DDB2 knockdown (Supplementary Fig. S5A-D). Side population (SP) cells, identified based on their ability to efflux the Hoechst 33342 fluorescent dye, have been shown to possess CSC properties (10). We then attempted to determine the effect of DDB2 on the percentage of SP cells. Different from above-detected ALDH+ and CD44+CD117+ populations, SP cells only account for a very small fraction of the total cancer cell population (less than 0.1%). Nevertheless, the overexpression of DDB2 reduced the SP fraction in one cell line (Supplementary Fig. S6A, B). Collectively, these results indicate that DDB2 is able to reduce the CSCs pool existing in ovarian cancer cells.

**Ovarian CSCs exhibit low DDB2 expression level**

To elucidate the mechanism of the DDB2-mediated regulation of the CSC population, we isolated the putative CSCs from various ovarian cancer cell lines, e.g., CP70, SKOV3, 2008, and 2008C13 based on the activity of ALDH, expression of specific surface markers CD44 and CD117, as well as the ability to form spheres and grow in non-adherent serum-free culture condition, respectively (9). The characteristics of isolated CSCs, including the ability to form tumor spheres in ultra-low attachment plates and the ability to form xenografts in immunodeficient mice at lower cell numbers, have been authenticated (Supplementary Fig. S7A-C; S8A-D; S9A-G). Both immunoblotting (Fig. 4A-D) and real-time PCR (Fig. 4E-F) analyses showed enhanced expression of Nanog, one of the self-renewal markers of stem cells and upregulated in various ovarian CSCs (5, 31), in these CSC populations in comparison to their parent bulk cancer cells, further supporting the stemness of these CSC populations. Interestingly, DDB2 protein level was found to decrease, while its mRNA level did not change in all CSC populations compared to their parent bulk cells (Fig. 4A-H). This data indicates that low DDB2 protein expression might be required for the maintenance of CSCs, while cellular DDB2 expression in CSCs is most likely regulated at post-transcription level.
DDB2 inhibits the self-renewal property and tumorigenicity of ovarian CSCs through suppressing NF-κB pathway

To further investigate whether low DDB2 expression is required to maintain the stem cell properties of ovarian CSCs, we transfected DDB2-expressing plasmids into three ovarian CSC populations and analyzed the expression of stem cell marker Nanog and their capability to form spheres in non-adherent culture condition. As shown in Fig. 5A-C, overexpression of DDB2 was accompanied by a reduced Nanog expression in all three CSCs. In addition, DDB2 overexpression significantly reduced the CSCs’ capability to form floating spheres in serum-free medium (Fig. 5D-F).

These data indicate that DDB2 is able to disrupt the self-renewal property of ovarian CSCs. Furthermore, we assessed the effect of DDB2 overexpression on the capability of CSCs to form xenografts in vivo. SKOV3-spheroids were transfected with either empty vectors or DDB2 expression plasmids, and $1 \times 10^4$ of these cells were injected into NOD/SCID mice subcutaneously. As shown in Fig. 5G & H, at 50 days post-injection, tumors can be found in 5/5 sites injected with vector-transfected SKOV3-spheroids, while only 2/5 sites injected with DDB2-overexpressing SKOV3-spheroids display visible tumor. In addition, the mean volumes of xenograft tumors generated from DDB2-overexpressing SKOV3-spheroids were significantly smaller than those arising from empty vector-transfected SKOV3-spheroids. Thus, DDB2 overexpression significantly inhibited the tumorigenicity of ovarian CSCs.

It has been recently reported that DDB2 is able to attenuate the activity of NF-κB through upregulating expression of IκBα in invasive breast cancer cells (23). Through the analysis of publically available datasets (www.cbioportal.org) (Supplementary materials and methods), we found that DDB2 mRNA expression level positively correlates with IκBα mRNA expression in ovarian cancer patients (Supplementary Fig. S10). Given NF-κB activation is required for the maintenance of CSCs in breast tumor (32-35), this prompted us to test whether DDB2 inhibits ovarian CSC population through enhancing IκBα expression. We thus determined the IκBα expression in DDB2-overexpressing and DDB2-downregulating ovarian cancer cell lines. As shown in Fig. 5I, DDB2 overexpression enhanced the protein level of IκBα, while DDB2-downregulation reduced the protein level of IκBα in the ovarian
cancer cell lines. We then transiently transfected DDB2 into 2008C13-CD44\(^+\)CD117\(^+\) cells and SKOV3-spheroids, and found that overexpression of DDB2 also increased the expression of \(I\kappa\Balpha\) in both ovarian CSC populations (Fig. 5J). This data indicates that DDB2-mediated reduction of ovarian CSC population could be attributed, at least in part, to the DDB2-induced upregulation of \(I\kappa\Balpha\), which results in the inactivation of NF-\(\kappa\)B pathway.

**Low DDB2 mRNA expression level correlates with poor prognosis of ovarian cancer patients**

Our data from the studies *in vitro* and *in vivo* indicates that loss of DDB2 is associated with highly tumorigenic potential in ovarian cancer cells. In order to understand whether there is any relationship between the level of *DDB2* expression and the prognosis in human ovarian cancer patients, we evaluated publicly available datasets for *DDB2* mRNA expression (Supplementary materials and methods). First, we used an online tool (http://kmplot.com) to assess the prognostic value of the Microarray-quantified *DDB2* expression level in ovarian cancer patients included in ten datasets (36). Kaplan-Meier survival curves showed that low *DDB2* mRNA expression correlates with poor overall and progression-free survival (PFS) among ovarian cancer patients (Fig. 6A,B). We then used another online tool cBioPortal (http://cbioportal.org) to analyze the effect of extremely low *DDB2* expression or high *DDB2* expression on the overall survival of ovarian cancer patients from The Cancer Genome Atlas (TCGA) data portal (37-39). Similarly, the Kaplan-Meier survival analyses demonstrated that ovarian cancer patients with *DDB2* mRNA expression Z-score less than -2 exhibited poor prognosis compared to those patients with *DDB2* expression Z-score greater than -2 (median survival months: 26.94 vs. 44.29), while the patients with *DDB2* expression Z-score higher than 0.5 exhibited better prognosis than those with *DDB2* expression Z-score less than 0.5 (median survival months: 55.88 vs. 41.53) (Fig. 6C,D). However, while the TCGA dataset demonstrates that overall survival is different, there is no difference in PFS. These analyses suggest that *DDB2* low expression is associated with poor outcome in ovarian cancer patients.
Discussion

Despite significant advances in diagnosing and treating ovarian cancer, one of the major clinical and scientific problems that remain unresolved is the prediction and inhibition of ovarian tumor recurrence after clinical remission. In the present study, we provide evidence showing that low DDB2 expression is correlated with poor outcome of ovarian cancer patients, overexpression of DDB2 in ovarian cancer cells reduces their tumorigenicity through limiting the CSC population, thus defining a novel function for DDB2 in the control of tumor relapse. DDB2 has been considered a tumor suppressor based on the findings that DDB2-/- mice were not only susceptible to UV-induced carcinogenesis, but also developed spontaneous malignant tumors at a high rate (40, 41). The analysis of publicly available datasets in this study indicates that low DDB2 mRNA expression correlates with poor outcome of ovarian cancer patients. Indeed, this kind of correlation can also be found in breast (23) and lung cancer patients (http://www.kmplot.com). Therefore, in combination with our findings in this study and others (19), we believe that DDB2 plays an important role in impeding tumor progression and tumor relapse.

DDB2 has been reported to inhibit metastasis of colon cancer (19) and limit the invasiveness of breast cancer (23). Mechanistically, DDB2 constitutively represses genes that are the key activators of EMT through its transcriptional regulation function in various colon cancer cell lines (19). In addition, DDB2 attenuates the activity of NF-κB by upregulating expression of IκB in breast cancer cells. EMT is believed to be a crucial mechanism for tumor metastatic progression (42), while NF-κB plays a causal role in migration and invasion of tumor cells and is required for maintenance of the malignant phenotype (43). Thus, the DDB2-dependent decrease of EMT and NF-κB activity could explain in part the correlation between high DDB2 expression and an improvement of the outcome in cancer patients. Besides these known mechanisms, we demonstrated for the first time in this study that DDB2 is able to reduce the abundance of CSCs in the bulk ovarian cancer cells, which provide a novel mechanism to explain the DDB2-mediated suppression of tumorigenicity. Our data demonstrated that the low expression of DDB2 is required for the maintenance of CSCs. Given that CSCs are believed to be the source of tumor
recurrence and metastasis, enhancement of DDB2 expression in ovarian tumors bears great potential in the improvement of the prognosis of tumor patients.

Mechanistic investigations demonstrated that overexpression of DDB2 reduces the expression of several CSCs self-renewal markers and their capability to form spheres in non-adherent condition. The maintenance and survival of CSCs are controlled by many pathways, such as Wnt, Notch and Hedgehog (44). It has been reported that Notch pathway in glioblastoma CSCs involves the constitutive activation of NFκB signaling, which upregulates Notch pathway genes, and promotes the survival of CSCs (45). In addition, mammary epithelial NF-κB is able to regulate the self-renewal of breast CSCs in Her2-dependent tumorigenesis (46). Given that DDB2 is able to upregulate the expression of IκBα, the inhibitor of NFκB, in breast tumor cells (23) and ovarian cancer cells, as well as ovarian CSCs, as demonstrated in the present study, we reason that DDB2 could suppress the survival of CSCs through downregulating the NFκB signaling. Moreover, NFκB is required for the induction and maintenance of the EMT (43), a process that can be used by cancer cells to reacquire “stemness”. Thus, it seems possible that DDB2 reduces the abundance of CSC through inhibiting NFκB-mediated EMT. Indeed, it has been reported that DDB2 is able to suppress EMT by constitutively repressing genes that are the key activators of EMT in colon cancer (24). However, we failed to find the alterations of epithelial marker E-Cadherin and mesenchymal marker Vimentin in ovarian cancer cells after DDB2 overexpression or knockdown. Therefore, we incline to the mechanism that DDB2 reduces the CSC subpopulation through shutting down the pathways required for the maintenance of CSCs.

The cellular level of DDB2 could be regulated at both transcript level and protein levels. According to the publicly available TCGA datasets, 16.5% (52/316) ovarian cancer patients display downregulated DDB2 mRNA (Z-score < -1), while 8.5% (27/316) display DDB2 mRNA upregulation (Z-score > 1) (www.cbioportal.org). Similarly, downregulation of the DDB2 mRNA can also be found in majority of the colon carcinoma datasets (24). In ovarian cancer datasets, heterozygous deletion contributes partially to the downregulation of DDB2 mRNA, while promoter CpG methylation seems not a determinant of DDB2 mRNA expression (Supplementary Fig. S11) (www.cbioportal.org). At the protein level, DDB2
can be ubiquitylated by DDB-Cul4A ubiquitin ligase, and its ubiquitylation and subsequent degradation are essential for its functions in NER (47, 48). DDB2 is also ubiquitylated in human cells in the absence of exogenous DNA damage (49), indicating that the steady-state level of DDB2 can be regulated by post-translational modification. Furthermore, DDB2 protein stability is reported to be regulated by USP24, a deubiquitylating enzyme, by removing the ubiquitin moiety from modified DDB2, thereby preventing DDB2 degradation (50). We found in this study that CSCs display reduced DDB2 protein level but not mRNA level in comparison with their parental bulk cancer cells. Thus, it is more likely that ovarian CSCs regulate the cellular DDB2 expression by decreasing its protein stability, and the underlying mechanisms warrants a further investigation in the future.

In conclusion, we report here a novel function of DDB2 in limiting tumorigenicity of ovarian cancer cells. Overexpression of DDB2 is able to inhibit the self-renewal ability of ovarian CSCs, probably through inhibiting NFκB pathway, leading to a reduction of CSC subpopulation, and finally results in an impediment of tumor progression.


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Authors’ Contributions

Conception and design: C.Han, Q.E.Wang

Development of methodology: C.Han, L.Gong, Q.E.Wang

Acquisition of data (provided animals acquired and managed patients, provided facilities, etc.): C.Han, R.Zhao, X.Liu, A.Srivastava, W.Zhao, M.Qu, Q.E.Wang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.Han, H.Mao, J.Yu, Q.E.Wang

Writing, review, and/or revision of the manuscript: C.Han, R.Zhao, J.Yu, Q.E.Wang

Study supervision: Q.E.Wang

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**Figure Legends**

**Figure 1.** Loss of DDB2 expression in ovarian cancer. DDB2 immunohistochemistry of human tissue microarray of normal ovary (n = 16), ovarian serous adenocarcinoma grade 1 (n = 4), grade 2 (n = 14), and grade 3 (n = 25). A, representative images of sections of each group are shown. B, intensity of DDB2 staining was scored from 1 to 4, and an individual value plot was generated to display the distribution of the intensity of DDB2 staining for normal ovary and ovarian cancer grade 1, 2 and 3. Bar: 95% CI for the mean. blue dot: median value. P value is calculated by Non-Parametrics Mann-Whitney test.

**Figure 2.** DDB2 represses tumorigenicity of ovarian cancer cells *in vitro* and *in vivo*. A, two clones of DDB2 stably overexpressing CP70 cells and two clones of DDB2 stably knockdown 2008 cells were selected, and DDB2 expression was determined. Exo-DDB2: Exogenous His-Xpress-tagged DDB2, End-DDB2: Endogenous DDB2. B-E, the DDB2-manipulated CP70 cells (B,D) and 2008 cells (C,E) were seeded in semisolid media containing serum-free DMEM/F12 supplemented with EGF and bFGF in Ultra-Low attachment plate and allowed to grow for 6 days. Representative images of colonies were shown (B,C). The number of colonies were counted and plotted (D,E). Bar: Standard deviation (SD), n = 6, *: P<0.01 compared with control cells. F,G, CP70-Vector and two DDB2-overexpressing CP70 cell lines were injected subcutaneously into nude mice, the tumors formed were counted after 4 weeks. Tumor sizes were measured (F), and tumors were removed from mice after 4 weeks (G). Bar: SD, *: P<0.01 compared with CP70-Vector.

**Figure 3.** DDB2 reduces the abundance of putative CSCs in ovarian cancer cell lines. A, ALDEFLUOR staining of CP70-Vector and two DDB2-overexpressing CP70 cell lines were conducted and analyzed by FACS. Number: percentage of ALDH^+^ cells. B, average percentage of ALDH^+^ cells in indicated cells. C, A2780 cells were transfected with DDB2 shRNA for 2 days. D, ALDEFLUOR staining of A2780 cells transiently transfected with either shControl or shDDB2 analyzed by FACS. E, average percentage of ALDH^+^ cells in indicated cells. Bar: SD, n=3, *: P<0.05; **: P<0.01 compared with A2780-shCtrl.
**Figure 4.** The expression of DDB2 protein, but not DDB2 mRNA, is reduced in ovarian CSCs. A-D, the protein level of DDB2 in various CSCs and their parental bulk cancer cells was determined using immunoblotting with anti-DDB2 antibody. The stem cell marker Nanog was also detected to show the CSC property. E-H, the mRNA level of DDB2 in various CSCs and their parental bulk cancer cells was determined using quantitative RT-PCR. The stem cell marker Nanog was also detected to show the CSC property. Bar: SD, n=3, **: P < 0.01 compared with bulk cancer cells.

**Figure 5.** Overexpression of DDB2 in ovarian CSCs alters the properties of CSCs. CP70-ALDH+ (A,D), SKOV3-spheroids (B,E), and 2008C13-CD44+CD117+ (C,F) cells were transfected with DDB2-expressing vector for 2 days. The expression of DDB2 and CSC marker Nanog were determined using immunoblotting (A-C). The sphere formation was assessed in non-adherent conditions (D-F). Bar: SD, n=3, **: P<0.01. G,H, NOD/SCID mice were subcutaneously injected with 1 × 10⁴ SKOV3-spheroids transiently transfected with either empty vectors or DDB2-expressing vectors. Representative image for tumor growth is shown (G). Tumor sizes were measured and the tumor growth was plotted (H), n = 5, bar: SD, *: P<0.05 compared with the vector group at the same time point. I, the expression of IκBα was determined in various ovarian cancer cell lines with either stable overexpression or knockdown of DDB2. J, 2008C13-CD44+CD117+ cells and SKOV3-spheroids were transiently transfected with DDB2-expressing plasmids for 2 days, the expression of IκBα was detected using immunoblotting.

**Figure 6.** Prognostic significance of DDB2 in ovarian cancer. A,B, the effect of DDB2 mRNA expression level on the overall survival (A) and progression-free survival (B) in 1,464 ovarian cancer patients was analyzed and the Kaplan-Meier plots were generated by Kaplan-Meier Plotter (http://www.kmplot.com). C,D, TCGA data was also analyzed to reveal the effect of extremely low DDB2 mRNA level (C) and high DDB2 mRNA level (D) on the overall survival of ovarian serous cystadenocarcinoma patients by using cBioPortal (http://cBioportal.org).
Fig. 1

A

DDB2

Normal

Grade 1

Grade 2

Grade 3

B

Intensity of DDB2 staining

Normal Grade 1 Grade 2 Grade 3

P=0.014

P=0.0005

P=0.0000
Fig. 2

### A

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- **Exo-DDB2**: 50 kDa
- **End-DDB2**: 37 kDa
- **Tubulin**: 37 kDa, 50 kDa

### B

- **CP70-Vector**
- **CP70-DDB2-1**
- **CP70-DDB2-3**

### C

- **2008-shCtrl**
- **2008-shDDB2-3**
- **2008-shDDB2-6**

### D

**Colony formation rate (%)**

- **CP70-Vector**
- **CP70-DDB2-1**
- **CP70-DDB2-3**

**Values**: 20 ± 2.3, 10 ± 2.2, < 10

* **p < 0.05**

### E

**Colony formation rate (%)**

- **2008-shCtrl**
- **2008-shDDB2-3**
- **2008-shDDB2-6**

**Values**: 30 ± 3.2, 20 ± 2.3, 10 ± 2.2

* **p < 0.05**

### F

**Tumour volume (cm³)**

- **CP70-Vector**: $(n=9)$
- **CP70-DDB2-1**: $(n=3)$
- **CP70-DDB2-3**: $(n=3)$

**Values**: 0 ± 0.4, 1 ± 0.3, 2 ± 0.1

* **p < 0.05**

### G

- **CP70**
- **CP70-DDB2-1**
- **CP70-DDB2-3**
**Fig. 3**

**A**

**DEAB**

**ALDEFLUOR**

**B**

![Bar graph showing ALDEFLUOR+ cells (%)](chart)

**CP70-Vector**

**CP70-DDB2-1**

**CP70-DDB2-3**

**C**

**A2780**

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**D**

**DEAB**

**ALDEFLUOR**

**E**

![Bar graph showing ALDEFLUOR+ cells (%)](chart)

**A2780-shCtrl**

**A2780-shDDB2**

*Denotes statistical significance.*
Fig. 4
Fig. 6

A

Low DDB2
High DDB2
HR = 0.73 (0.63 – 0.85)
Logrank test p-value: 0.00004

B

Low DDB2
High DDB2
HR = 0.83 (0.72 – 0.96)
Logrank test p-value: 0.011

C

DDB2 mRNA z-score < -2
DDB2 mRNA z-score > -2
Logrank test p-value: 0.01048

D

DDB2 mRNA z-score < 0.5
DDB2 mRNA z-score > 0.5
Logrank test p-value: 0.029866
Molecular Cancer Research

DDB2 Suppresses Tumorigenicity by Limiting the Cancer Stem Cell Population in Ovarian Cancer

Chunhua Han, Ran Zhao, Xingluo Liu, et al.

Mol Cancer Res  Published OnlineFirst February 26, 2014.

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