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Biological Significance of HORM-A Domain Containing Protein 1 (HORMAD1) in Epithelial Ovarian Carcinoma

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Abstract

The present study was undertaken to determine the expression and biological significance of *HORMAD1* in human epithelial ovarian carcinoma. We found that a substantial proportion of human epithelial ovarian cancers expressed *HORMAD1. In vitro, HORMAD1* siRNA enhanced docetaxel induced apoptosis and substantially reduced the invasive and migratory potential of ovarian cancer cells (2774). *In vivo*, *HORMAD1* siRNA-DOPC treatment resulted in reduced tumor weight, which was further enhanced in combination with cisplatin. *HORMAD1* gene silencing resulted in significantly reduced VEGF protein levels and microvessel density compared to controls. Our data suggest that *HOMRAD1* may be an important therapeutic target.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

Keywords

HORMAD1; ovarian cancer; angiogenesis; VEGF; xenograft

1. INTRODUCTION

Ovarian cancer is the sixth most common cancer in women and is the leading cause of female mortality due to gynecologic malignancies [1]. Currently, the mainstay of treatment for primary disease is cytoreductive surgery followed by 6 cycles of platinum based chemotherapy [2; 3]. Despite advances in surgery and chemotherapy and high response rates early in the course of disease, majority of patients develop recurrent cancer that becomes refractory to chemotherapy and leads to death. Second line anti-neoplastic agents only yield responses in 10–25% of patients [4; 5; 6]. Therefore the need to identify novel targets and develop new therapeutic approaches is both essential and urgent.

HORMA domain containing proteins (HORMAD) are known to play a role in cell-cycle regulation. For example, they can act as adaptor proteins to recruit other proteins to check-point regulation and DNA-repair in mitosis and meiosis [7; 8]. HORMAD proteins (HORMAD 1 and 2) have been identified in mammalian and mouse developing gonads with expression restricted to germ cells [8; 9; 10]. Recently, serial analysis of gene expression (SAGE) in gastric cancer reveled that HORMAD1 was over expressed in >45% of gastric cancers specimens tested [11]. HORMAD1 has been shown to be expressed in basal and luminal breast cancers and was considered to be a potentially important oncogene [12]. However, the expression and clinical significance of HORMAD1 in epithelial ovarian cancer is not known, and is the focus of the current study.

2. MATERIALS AND METHODS

2.1 Cell culture and conditions

Non-transformed ovarian epithelial (HIO-180) and ovarian cancer (HeyA8, SKOV3ip1, and A2780, HeyA8-MDR, SKOV3-TR, ES2 and 2774) cell lines were maintained and propagated *in vitro*, as previously described [13; 14]. These cells were acquired from the characterized cell line core at the U.T.M.D Anderson Cancer Center (Houston, TX). Briefly, we maintained HeyA8 and SKOV3ip1, A2780 and 2774 in RPMI 1640 supplemented with 15% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bio-products, Woodland, CA), and HeyA8-MDR and SKOV3-TR in RPMI 1640 supplemented with 15% FBS, 300ng/mL paclitaxel, and 0.1% gentamicin sulfate. All cells were kept in 5% CO_2/95% air at 37°C.

2.2 Western blot analysis

Western blot analysis was performed as described previously.[15] Briefly, lysates from cultured cells were prepared with modified RIPA buffer and protein concentrations were determined with a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL). Lysates were separated using 8% sodium dodecyl sulfate – polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane by semidry electrophoresis (Bio-Rad Laboratories, Hercules, CA), blocked with 5% milk and incubated at 4 °C with primary antibody (against HORMAD1, TNF-α, and NF-κB [Abcam, Cambridge, MA]), after washing with TBST, the membranes were incubated with 1 μg/mL horseradish peroxidase (HRP) – conjugated horse anti-mouse IgG (Amersham, Piscataway, NJ). HRP was visualized by use of an enhanced chemiluminescence detection kit (Pierce). To confirm equal sample loading, the blots were stripped and re-probed with an antibody specific for β-actin (0.1 μg/mL; Sigma).

2.3 Enzyme-linked immunosorbent assay (ELISA)

Vascular endothelial cell growth factor (VEGF) protein levels in the supernatants and lysates from ovarian cancer cell line (2774) that were treated with either control siRNA or HORAMD1 siRNA (in triplicates) were quantified by ELISA, using the quantikine Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol and as previously described [16]. Average protein from experiments performed in triplicates is presented.

2.4 Animals, Orthotopic *in vivo* Model and Tissue Processing

Female athymic nude mice were purchased from the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD). These animals were cared for according to guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service Policy on Human Care and Use of Laboratory Animals. All mouse studies were approved and supervised by the M. D. Anderson Cancer Center Institutional Animal Care and Use Committee. The ovarian cancer cells (2774) were trypsinized, washed and resuspended in Hanks' balanced salt solution (Gibco, Carlsbad, CA) and injected into mice (2774: 1×10^6 cells/animal). Seven days after the tumor cell injection, mice were randomly divided and treated with siRNA incorporated in 1,2-dioleoylsn-glycero-3-phosphatidylcholine (DOPC) neutral nanoliposomes (intraperitoneal [IP] administration) with or without cisplatin, according to the following groups (n=10/group): control siRNA-DOPC, HORMAD1 siRNA-DOPC, control siRNA-DOPC + cisplatin, and HORMAD1 siRNA-DOPC + cisplatin. Twice weekly treatments continued for 4 weeks, after which, all mice were euthanized, necropsied, and tumors were harvested. Tumor weights, number and location of tumor nodules, and quantity of ascites were recorded. Tumor tissue was fixed in formalin for paraffin embedding, and frozen in optimal cutting temperature (OCT) media to prepare frozen slides.

2.5 Small interfering RNA (siRNA) preparation

In order to down-regulate *HORMAD1* gene *in vitro* and *in vivo,* HORMAD1 specific siRNA was utilized. Non-targeting, nonspecific sequence 5′-ATTTCTCCGAACGTGTCACGT-3′ was used as control. All siRNAs were purchased from Sigma-Aldrich and prepared as previously described [17; 18]. The lyophilized DOPC incorporated siRNA was hydrated with PBS, and injected IP twice weekly following our previously published protocols [19] at 5.0 μg siRNA/200 μL suspension per animal.

2.6 CD31 Staining

CD31 staining on fresh frozen sections of tumor tissues from the therapy experiment (2774 model) was performed as described previously [15; 20]. Briefly, slides were fixed in cold acetone for 10 minutes and did not require antigen retrieval. Endogenous peroxide was blocked by adding 3% H_2O_2 in methanol for 8 minutes, and after washing, the nonspecific proteins were blocked using 5% normal horse serum and 1% normal goat serum in PBS for 15 minutes at room temperature. Slides were incubated with primary antibody CD31 (Pharmingen, San Diego, CA) in blocking solution overnight at 4 °C. After washing with PBS, the appropriate HRP-conjugated secondary antibody in blocking solution was added for 1 hour at room temperature. Slides were counterstained (blue nuclei) with Hoechst (1:10,000). Fluorescence microscopy was used to analyze slides at 200x. To quantify MVD, the microvessels within five randomly selected 0.159-mm^2 fields at x200 were counted for each sample, a single microvessel was defined as a discrete cluster or at least 3 cells which stained positive for CD31 ($CD31^+$; red).

2.7 TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed on fresh frozen tumor tissue (n=5 per group) using Promega Kit (Promega, Madison, WI) as described previously [21]. To quantify apoptotic cells, the number of TUNEL positive (green) cells were counted (and divided by the total number of cells in each field and multiplied by 100) in at least 3 random fields at 200X magnification, thus reported as percent TUNEL positive cells.

2.8 Patient Samples

After Institutional Review Board approval for this study, archived, fresh frozen samples were obtained from 90 patients with serous epithelial ovarian carcinoma who underwent surgery at the University of Texas M.D. Anderson Cancer and who had adequate tissue available for mRNA evaluation.

2.9 RNA Extraction and cDNA

Approximately, 35 mg of fresh frozen tumor was obtained from the U.T.M.D. Anderson tumor bank from the ovarian cancer specimens. After freezing the specimen with liquid nitrogen, mortal and pestle was used to grind the samples and 1.0 mL of Trizol (Invitrogen) was homogenized with the tumor. 200 μL of chloroform was added and sample centrifuged at 12000 G. After chloroform extraction, total-RNA was precipitated with 500 μL of isopropanol followed by a 75% ethanol wash. RNA was then air-dried and dissolved in Rnase-Free water and stored at −80 °C. RNA quality was confirmed and only RNA with greater than 1.5 OD ratio (260/280) was used to make the complementary DNA (cDNA). The cDNA was generated with 2.0 μg of RNA using SuperScript-II reverse transcriptase kit (Invitrogen) as previously described [14].

2.10 Real-Time quantitative PCR

HORMAD1 gene specific primers (5′-TGTTTGTCACCTACACTCAGG-3′ and 5′-GTAAGGAAGAAGAAACTATGC-3′) were designed and purchased from Sigma-Aldrich. Primers where diluted in Rnase free water according to the manufacturers recommendations. Quantitative RT-PCR was performed in Applied Biosystems 9500 series using conditions that have been previously described [14]. Beta-actin was used as endogenous control for each reaction, while normal ovarian cDNA was used as internal reference. Each reaction was repeated three times. HORMAD1 expression was reported by the Applied Biosystems software as RQ values (average fold-change compared to normal ovary). The cycling conditions were: 94°C for 3 min; 94°C for 30 s, 54°C for 30 s, and 72°C for 25 s for 30 cycles; and 72°C for 7 min.

2.11 Microarray analysis

cDNA microarray was performed using the Illumina platform on the 2774 ovarian cancer cells that were treated in 10 cm cell-culture plates with either *HOMRAD1* (8.0μg) or control siRNA (8.0 μg) in triplicate using RNAiFect transfection reagent (Qiagen; Valencia, CA) per manufacturer's recommendations. *HOMRAD1* gene silencing was confirmed using qRT PCR prior to the microarray analysis. Gene expression data from microarray analysis was then loaded into the IPA Ingenuity pathway data base and differentially expressed genes related to apoptosis were analyzed.

2.12 Statistical Analysis

Continuous variables were compared using Student's *t* test (between two groups) or analysis of variance (for all groups) if normally distributed. In nonparametric values, continuous variables were compared with the use of the Mann-Whitney U test or Kruskal-Wallis test

(for all groups). The statistical significance of the data was determined by using the Statistical Package for Social Scientists software (SPSS, Inc., version 17.0, Chicago, IL).

3. RESULTS

3.1 HORMAD1 expression in epithelial ovarian cancer cells

Using RT-PCR, we first examined HORMAD1 mRNA expression in multiple ovarian cancer cell lines (Figure 1A). We specifically tested a non-transformed epithelial ovarian cancer cell line (HIO-180), several chemosensitive (A2780-Par, HeyA8, SKOV3ip1), two chemoresistant (SKOV3-TR and HeyA8-MDR), and two ascites producing (ES2 and 2774) epithelial ovarian cancer cell lines. Five out of seven ovarian cancer cell lines tested, expressed *HOMRAD1* gene. Additionally, Western blots demonstrated similar trends in HORMAD1 protein in ovarian cancer cell lines (Figure 1A).

Next, we performed quantitative real-time PCR in 90 human epithelial ovarian cancer samples and determined the level of expression of HORMAD1 mRNA compared to normal ovarian surface epithelium (OSE). Histogram with distribution of *HORMAD1* transcript in human epithelial ovarian cancer sample is shown in Figure 1B. Median fold- change of *HORMAD1* was 5.6-fold (range 0.23–1297.5) compared to the normal OSE. Additionally, 76.1% of the ovarian cancer samples had increased expression of *HORMAD1* compared to normal OSE.

3.2 Effect of anti-HORMAD1 siRNA on ovarian cancer cell survival

Due to the potential oncogenic role of *HORMAD1* in breast carcinoma and increased expression of *HORMAD1* seen in our human epithelial ovarian cancer samples as well as in ovarian cancer cell lines, we wondered whether *HORMAD1* expression was important for ovarian cancer cell survival. Here we selected an ovarian cancer cell line with highest *HORMAD1* expression (2774) and asked whether targeting *HORMAD1* will decrease ovarian cancer cell viability and potentially add to the efficacy of chemotherapy (Figure 1C). Exposure to docetaxel for 48 h resulted in 1.4-fold increase in apoptosis compared to control. While *HORMAD1* silencing alone had no effect on 2774 cell survival the combination of *HORMAD1* siRNA and docetaxel treatment resulted in 2.7-fold increase in apoptosis compared to control. Here, the addition of *HORMAD1* silencing resulted in 53% higher apoptosis compared to docetaxel treatment alone ($p<0.05$). Similar experiments were repeated in another ovarian cancer cell line (SKOV3) that does not express HORMAD1. As expected, compared to control siRNA, HORMAD1 siRNA did not have any effect on cell survival (Figure 1C).

3.3 Effect of anti-HORMAD1 siRNA on ovarian cancer cell invasion and migration

To determine whether HORMAD1 played a significant role in the invasive or migratory potential of ovarian cancer cells, we used *in vitro* assays. Following treatment with *HORMAD1* specific siRNA (24 h), we measured the total number of invasive and migratory cells exposed to anti-HORMAD1 or control siRNA (Figure 1D). HORMAD1 siRNA reduced 2774 ovarian cancer cell invasion by 65% and migration by 42% (both, $p<0.05$) as compared to control (scrambled) siRNA.

3.4 Effect of anti-HORMAD1 siRNA silencing on ovarian cancer growth

Considering the effects of HORMAD1 silencing on cell viability as well as on invasion and migration, we next tested the therapeutic effects of silencing *HOMRAD1* gene *in vivo* (Figure 2A). We utilized a well-characterized orthotopic ovarian cancer model (2774) and used *HORMAD1* specific siRNA incorporated into DOPC nanoliposomes with or without cisplatin chemotherapy. *HORMAD1* siRNA or cisplatin monotherapy resulted in 45% and

81% reduction in tumor weight ($p<0.05$, both), respectively. Combination treatment resulted in the greatest reduction in tumor weight (by 94%, $p<0.01$) compared to control treatment and cisplatin monotherapy (by 72%, $p<0.05$). Similar results were noted with tumor nodules (data not shown). *HORMAD1* siRNA-DOPC or cisplatin monotherapy resulted in 25% and 88% reduction in number of mice with measureable ascites ($p<0.05$; Figure 2B) compared to control siRNA-DOPC treatment. The combination of cisplatin and HORMAD1 siRNA-DOPC resulted in complete absence of ascites.

3.5 Effect of HORMAD1 targeting on tumor microenvironment

To understand the biological effects of *HORMAD1* gene silencing, a series of *in vitro* and *in vivo* experiments were carried out. First, to determine the effects of *HOMRAD1* silencing on tumor associated angiogenesis, we performed CD31 staining on fresh frozen tumor samples from all four treatment groups (Figure 2C). HORMAD1 siRNA-DOPC or cisplatin alone resulted in 38–62% reduction ($p<0.05$, both) in MVD, and the combination resulted in 75% reduction ($p<0.05$) compared with controls.

Given that our *in vitro* studies pointed towards a decrease in cell survival as a possible mechanism by which *HORMAD1* exerts its effect on tumor cells and that a reduction in tumor weight was seen in groups that received HORMAD1 siRNA-DOPC treatment, we also performed TUNEL staining (Figure 2C) to assess effects on tumor cell apoptosis. Cisplatin monotherapy resulted in 15-fold increase in apoptosis compared to controls ($p<0.05$). Combination treatment with HORMAD1 siRNA-DOPC and cisplatin resulted in a substantial (by 23-fold) increase in tumor cell apoptosis compared to the controls ($p<0.05$) and a 36% additional increase in apoptosis compared to cisplatin monotherapy ($p<0.05$). Furthermore, to analyze the effects of HORMAD1 silencing on VEGF protein levels in ovarian cancer cell (2774), we silenced HORMAD1 using specific siRNA and subject ovarian cancer cells to ELISA. HORMAD1 silencing resulted in significant reduction (by 22%) in VEGF levels compared to controls ($p<0.05$). We also measured VEGF levels in the supernatant, and there was a 28% decrease in VEGF levels following HORMAD1 silencing compared to controls ($p=0.02$).

3.6 Markers of HORMAD1 silencing in ovarian cancer cells

To identify potential markers of response to HORMAD1 gene silencing, we performed genomic analyses on control *versus* HORMAD1siRNA treated 2774 ovarian cancer cells (Figure 3A). There were 192 genes that were differentially expressed between control siRNA and HORMAD1 siRNA treatment groups ($p<0.001$). Since our biological data pointed toward apoptosis and impaired cell survival as the dominant effects following HORMAD1 silencing, we focused on genes in these pathways. There were 21 genes related to these pathways that were significantly altered following HORMAD1 gene silencing (Figure 3B), including NF-κB and TNF-α. Next we analyzed the effects of HORMAD1 silencing on NF-κB and TNF-α at the protein level using western blot (Figure 3C). Forty-eight hours after HORMAD1 silencing using specific siRNA in ovarian cancer cell line (2774), we found substantial reduction in NF-κB and TNF-α.

4. DISCUSSION

The key findings of this study are that *HORMAD1* is overexpressed in a substantial proportion of human epithelial ovarian cancers compared with normal ovarian tissues. HORMAD1 targeting *in vivo* with siRNA-DOPC substantially reduced ovarian cancer growth and ascites production. These biological effects were associated with reduction in angiogenesis and cell survival, and substantial reduction in VEGF and NF-κB.

HORMA domain family of proteins are known to regulate cell cycle and play key functions in mitosis and meiosis [7; 8; 22]. Developmentally, *HORMAD1* is expressed during gonadal development and plays a crucial role in meiosis I. *HORMAD1* is shown to be upstream of ATM kinase (a serine-threonine protein kinase) activation and lack of HORMAD1 is reported to disrupt ATM autophosphorylation [8]. A reduction in ATM activation is suggested to be associated with cell cycle regulation, apoptosis, and response to DNA damage repair, while ATM activity is linked to increased auto-phosphorylation of ATM at several sites [23]. Our findings of increased apoptosis, and enhanced anti-cancer activity of docetaxel in combination with HORMAD1 silencing may be explained by our observation of reduction in NF-κB protein levels after HORMAD1 silencing as well as the known inhibitory role of HORMAD1 on ATM kinase activation.

Tumor growth largely depends on increased nutrient supply and oxygen delivery and requires corresponding increases in angiogenesis [24; 25]. VEGF released by the tumor cells acts as a key element in induction of angiogenesis. Like many solid tumors, ovarian cancer has significantly increased expression of VEGF [26] and over expression of VEGF is biologically known to be related to the formation of ascites [27]. In this study, we show that HORMAD1 silencing results in substantial reduction in MVD and ascites formation. These findings are supported by our observation that HORMAD1 silencing also reduced VEGF protein levels in ovarian cancer cells.

Recent studies describe an increased expression of *HORMAD1* in breast and gastric carcinomas [11; 12]. *HORMAD1* has been identified as a potential oncogene in breast carcinoma subtypes [12]. *HORMAD1,* therefore, belongs to a group of germ cell genes that are expressed in cancer and better known as cancer/testis antigens. Several cancer testis antigens have been shown to be expressed in ovarian cancer [28; 29]. These antigens, including HORMAD1, are exclusively expressed in germ cells. Such restricted expression in normal tissues, may allow for tumor specific therapies with little side effects. Additionally, recent developments in small interfering RNA technology [16; 18; 30], may allow relatively specific targeting of *HORMAD1* transcripts in ovarian carcinoma and may have therapeutic implications for ovarian cancer management.

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Abbreviations

HORMAD1	HORMA domain containing protein 1
FBS	fetal bovine serum
MVD	mean vessel density

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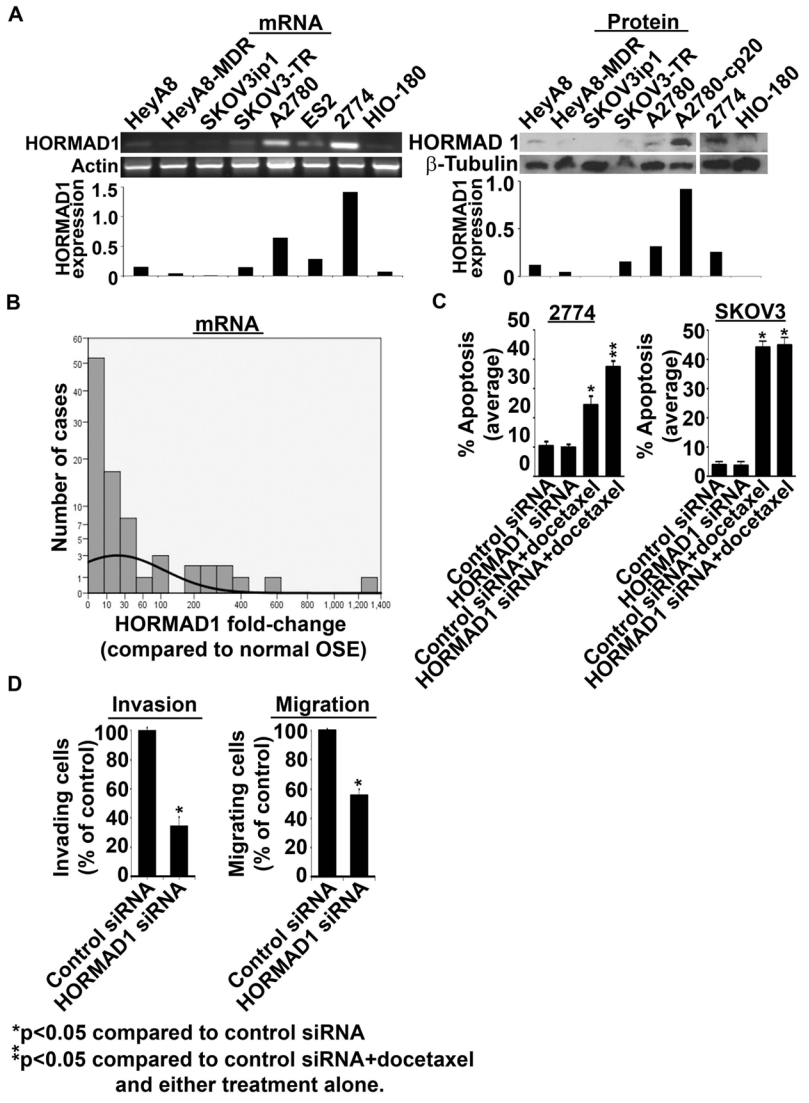

Figure 1. Characterization and significance of HORMAD1 in ovarian cancer cells

A) RT-PCR and Western blot analysis was performed to determine the mRNA and protein expression of HORMAD1 in ovarian cancer cell lines. β-actin and β-tubulin were used as loading control. Relative expression densities are shown in respective bar-graphs B) Histogram with distribution of HORMAD1 in human ovarian cancer samples, results represented from 90 patient's tumor samples. X-axis, *HORMAD1* fold- change (power scale); Y-axis, number of cases (power scale); Gray bar, number of case for *HORMAD1* fold-change; and black curve, distribution of *HORMAD1*. Distribution of *HORMAD1* is skewed (Median change, 5.6-fold, range 0.23–1297.5; Mean change, 52.8-fold, SD 161.4; skewness 5.8, $p<0.001$). Increased HORMAD1 fold change (≥1) counted 76.1%. C) Effect of HORMAD1 silencing on apoptosis. *HORMAD1* was silenced using HORMAD1 specific siRNA in 2774 and SKOV3 ovarian cells with and without docetaxel treatment and level of apoptosis was assessed using flow cytometry (Annexin V). Average percent apoptosis (Annexin V/PE positive cells) are reported. D) Effect of HORMAD1 silencing on invasion and migration. Twenty-four hours after transfecting 2774 ovarian cancer cells with either HORMAD1 siRNA or control siRNA, invasion and migration assays were performed. The percent of invasive or migratory cells compared to controls were determined with light

microscopy after fixation and staining. Each experiment was performed in triplicate. Error bars represent S.E.M

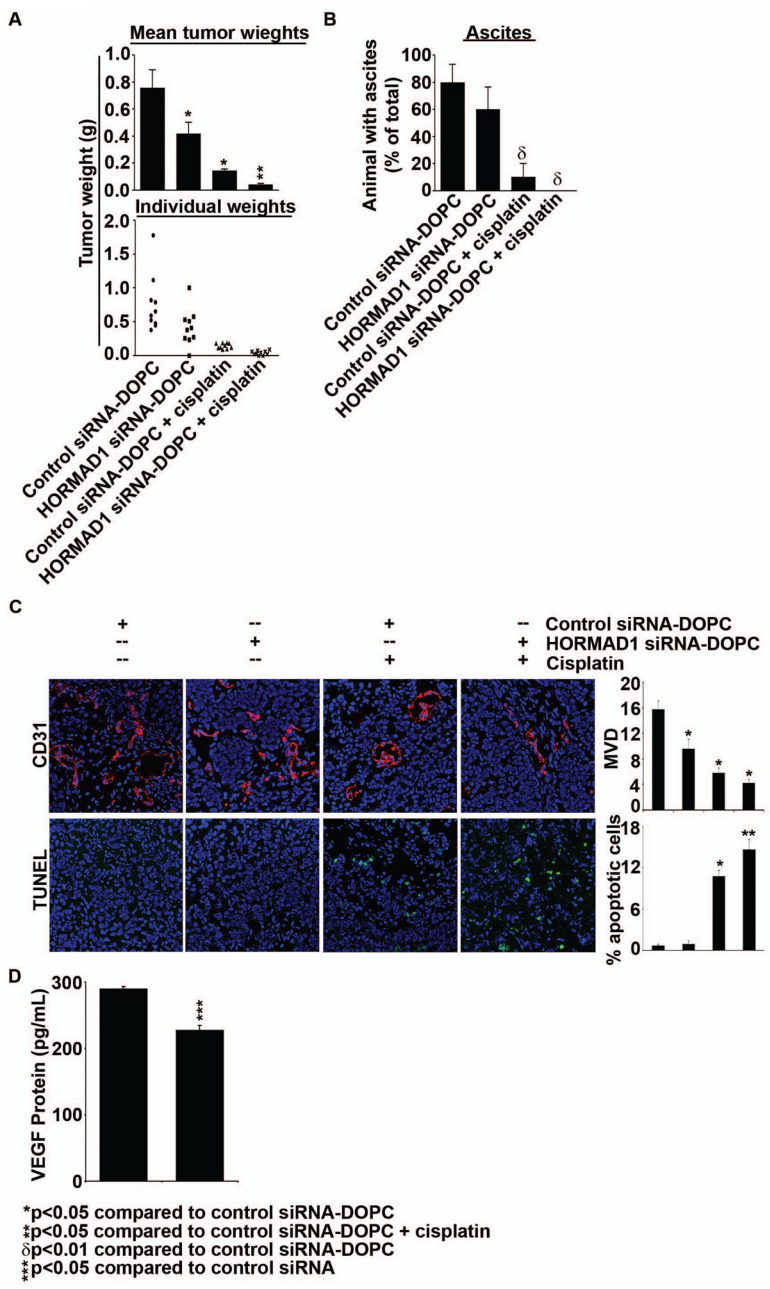

Figure 2. Effects of HORMAD1 silencing on ovarian cancer growth

In vivo efficacy of HORMAD1 siRNA-DOPC was evaluated in nude mice using the orthotopic 2774 ovarian cancer model. *HOMRAD1* silencing was achieved with specific siRNAs incorporated into neutral nanoliposomal DOPC, injected twice weekly with or without cisplatin. At the end of the study, mice were sacrificed and tumors were harvested. A) average tumor weight (bar graph) and individual weight distribution (dot graph) is shown. B) Percent of total number of animal with ascites in all treatment groups. C) Effect of HORMAD1 siRNA-DOPC on tumor microenvironment. Tissues harvested following HORMAD1 siRNA-DOPC therapy were subjected to fluorescence staining, angiogenesis (CD 31) and apoptosis (TUNEL). CD31 (mouse endothelial cells, red; tumor cell nuclei, blue) staining is shown at the 200X magnification. The graphs on the right shows mean

number of microvessel density (MVD) from each treatment group. Average MVD was calculated by averaging MVD counts from 5 random fields per slide, and at least 3 slides were examined for each treatment group. For the TUNEL stain, 5 random fields per slide were examined with fluorescence microscopy and the number of apoptotic bodies (green) and nuclei (blue) are reported as average percent apoptotic cells (200X) on the graph on the right. D) After HORMAD1 silencing in ovarian cancer cells (2774), VEGF protein levels were measured using ELISA in triplicate. Bars represent average amount of VEGF in control vs. HORMAD1 siRNA treated cell. Error bars represent S.E.M.

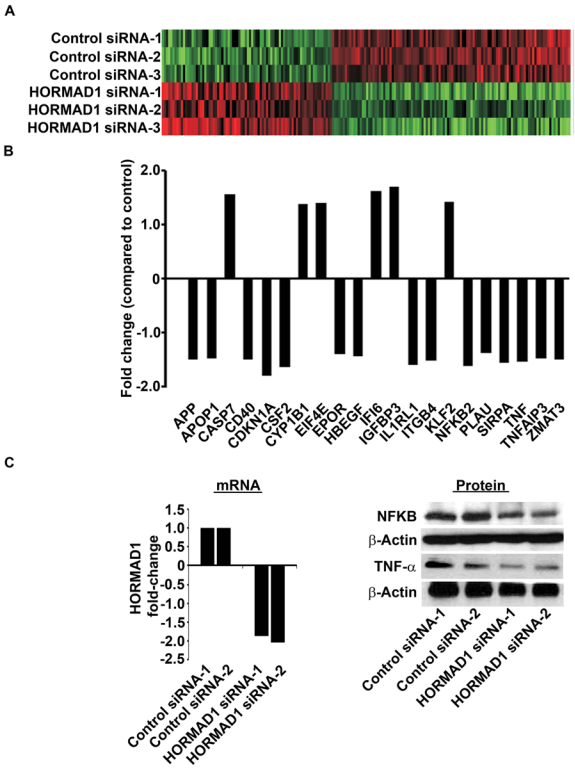

Figure 3.

Effect of *HORMAD1* silencing on gene expression profile of ovarian cancer cells. After *HORMAD1* silencing with siRNA (*in vitro*), microarray analysis was performed. Heatmap (A) represents the over all gene expression changes. B) Apoptosis related genes that were differentially expressed between control siRNA and HORMAD1 siRNA treatment groups. C) HORAMD1 mRNA expression is shown as fold change after treatment of 2774 ovarian cancer cells with control siRNA or HORMAD1 specific siRNA. Expression of NF-κB and TNF-α protein is analyzed in control and HORMAD1 siRNA treated ovarian cancer cell (2774) in duplicate. β-actin is used as a loading control.