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# HE4 (WFDC2) gene overexpression promotes ovarian tumor growth

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**Selective overexpression of Human epididymal secretory protein E4 (HE4) points to a role in ovarian cancer tumorigenesis but little is known about the role the HE4 gene or the gene product plays. Here we show that elevated HE4 serum levels correlate with chemoresistance and decreased survival rates in EOC patients. HE4 overexpression promoted xenograft tumor growth and chemoresistance against cisplatin in an animal model resulting in reduced survival rates. HE4 displayed responses to tumor microenvironment constituents and presented increased expression as well as nuclear translocation upon EGF, VEGF and Insulin treatment and nucleolar localization with Insulin treatment. HE4 interacts with EGFR, IGF1R, and transcription factor HIF1α. Constructs of antisense phosphorothio-oligonucleotides targeting HE4 arrested tumor growth in nude mice. Collectively these findings implicate increased HE4 expression as a molecular factor in ovarian cancer tumorigenesis. Selective targeting directed towards the HE4 protein demonstrates therapeutic benefits for the treatment of ovarian cancer.**

Human epididymis protein 4 (HE4), also called whey-acidic-protein (WAP) four-disulfide core domain protein 2 (WFDC2) was initially described to have tissue specific expression in the epididymis[1]. Clinical research in the last decade revealed that HE4 is expressed in a limited number of other organs, including the female reproductive tract, breast tissue, kidney, regions of the respiratory tract and nasopharynx[2–4]. HE4 in human ovarian cancer cells is produced as a ~13 kD protein and converted to a ~25 kD secreted glycosylated protein. HE4 (WFDC2) is highly overexpressed in epithelial ovarian cancer (EOC)[5–8] compared to normal ovarian epithelium and the measurement of serum HE4 levels in women with EOC has been shown clinical relevance. The USFDA cleared HE4 as a biomarker for the detection of ovarian cancer in women presenting with an ovarian cyst or pelvic mass as part of the Risk of Ovarian Malignancy Algorithm (ROMA) and for monitoring women diagnosed with EOC[9–13]. Overexpression of Human epididymal secretory protein E4 (HE4) in EOC points to a role in ovarian cancer tumorigenesis, however little is known about the biological functions of the HE4 gene or its gene product. Here we show that elevated HE4 serum levels correlate with chemoresistance and decreased survival rates in EOC patients and demonstrate that HE4 overexpression promotes ovarian tumor growth in an animal model. We also demonstrate that HE4 interacts with growth factors and oncogenes previously linked to ovarian tumor growth and chemoresistance. Finally, we show that antisense inhibition of HE4 via novel phosphorothio-oligonucleotides (PTOs) resulted in reduced ovarian cancer cell viability and suppressed growth of xenografted tumors in mice. Taken together, our studies provide evidence that HE4 overexpression plays an important role in ovarian tumor growth and chemoresistance.

## Results

**HE4 expression levels correlate with lower survival and chemoresistance in human ovarian cancer patients.** To delineate the correlation of serum HE4 levels and chemoresistance in women with EOC we investigated the association of pre-operative serum HE4 levels with chemosensitivity and survival in a retrospective study of 89 women with EOC at Women and Infants Hospital (Institutional Review Board approval:11–005). Patients were stratified based on preoperative serum HE4 levels. Survival curves were plotted and Cox hazards regression

analysis was used to determine association between prognostic variables and overall survival (OS). The median OS at 5 years was 53.9%. Women with a serum HE4 level ≥500 pM had a 5-year OS of 27% compared to 59% for those with HE4 <500 pM (p=0.005) (Fig. 1A). Median OS analysis for high HE4 versus low HE4 expressers revealed a Hazard Ratio (HR) of 2.2 (95% CI: 1.3 – 3.9; p=0.005). Examination of the Risk of Ovarian Malignancy Algorithms (ROMA) scores, which employs serum levels of HE4 and CA125 along with menopausal status to predict the presence of ovarian cancer, showed that women with a ROMA score ≥60% had a 5-year survival rate of 38% and those with a ROMA score <60% had a 76% 5-year survival rate (p=0.003). Likewise analysis of median OS provided a HR of 3.3 (95%CI: 1.4–7.9%; p=0.0014) (Fig. 1B). In correlation with these findings, patients with platinum resistant disease had a 5 years survival rate of 29% compared with 57% for patients with platinum sensitive disease (p=0.015). Analysis of median OS in the platinum resistant group versus the platinum sensitive group provided a HR of 2.0 (95%CI: 1.3–3.8; p=0.0068). Within two years, the platinum resistant group witnessed a threefold higher death rate of 52% compared with the platinum sensitive group with a rate of 14% (p=0.001) (Fig. 1C). In concordance with our observations of the correlation between HE4 over expression and poor patient survival rates, CA125 overexpression with serum levels ≥100 U/ml was also associated with lower 5 year survival rates compared with patients that had serum levels < 100 U/ml (58 vs 41%). Median OS analysis provided a HR of HR:1.9 (95%CI: 1.0–3.6; p=0.046) (Fig. 1D). However, HE4 and ROMA scores emerged as more sensitive predictors of survival and chemoresistance than CA125 alone. Similarly, stage (stage I & II versus stage III & IV) [HR 4.4 (95% CI: 1.7–11.0), p=0.0002], and postmenopausal vs menopausal [HR: 3.0, (95% CI: 0.9–9.6), p=0.0297] was also associated with higher risk of death and poor survival (Supplementary Fig. 1).

**Stable HE4 overexpression promotes cisplatin resistance in ovarian cancer cells and tumor growth in nude mice.** To study the biological role of HE4 overexpression in ovarian cancer tumorigenesis and chemoresistance, stable HE4 overexpressing SKOV-3 (HE4C1, HE4C7) and OVCAR-8 (HE4C5) ovarian cancer cell clones were developed (see methods). OVCAR-8 and SKOV-3 cells are both derived from ovarian epithelial adenocarcinoma and are resistant to platinum based chemotherapeutics which are the standard of care for ovarian cancer treatment. HE4 levels were measured in cell lysates and media (Fig. 2A). Evaluation of the cell

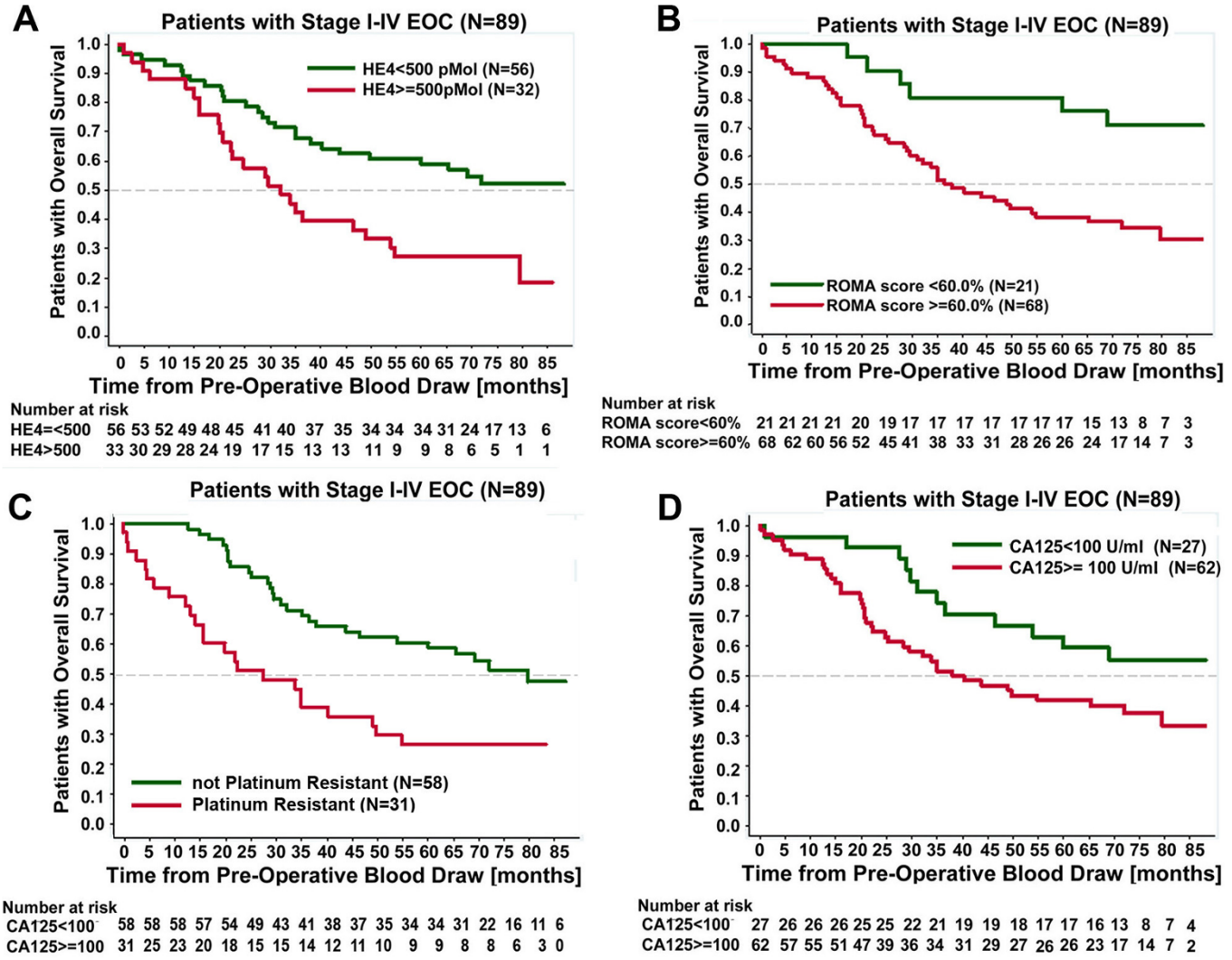


**Figure 1 | Survival analysis of ovarian cancer patient cohort.** (A) Kaplan-Meier survival curve of 89 ovarian cancer patients (all stages) show decreased survival for patients with HE4>500 pM compared with patients with HE4<500 pM. (B) Patients with ROMA scores >60% have decreased survival compared with patients that have ROMA scores <60%. (C) Clinical assessment showing Platinum resistant tumors affect patient survival. (D) Patients with CA125 >100 U/ml experience higher mortality than those with CA125 <100 U/ml.

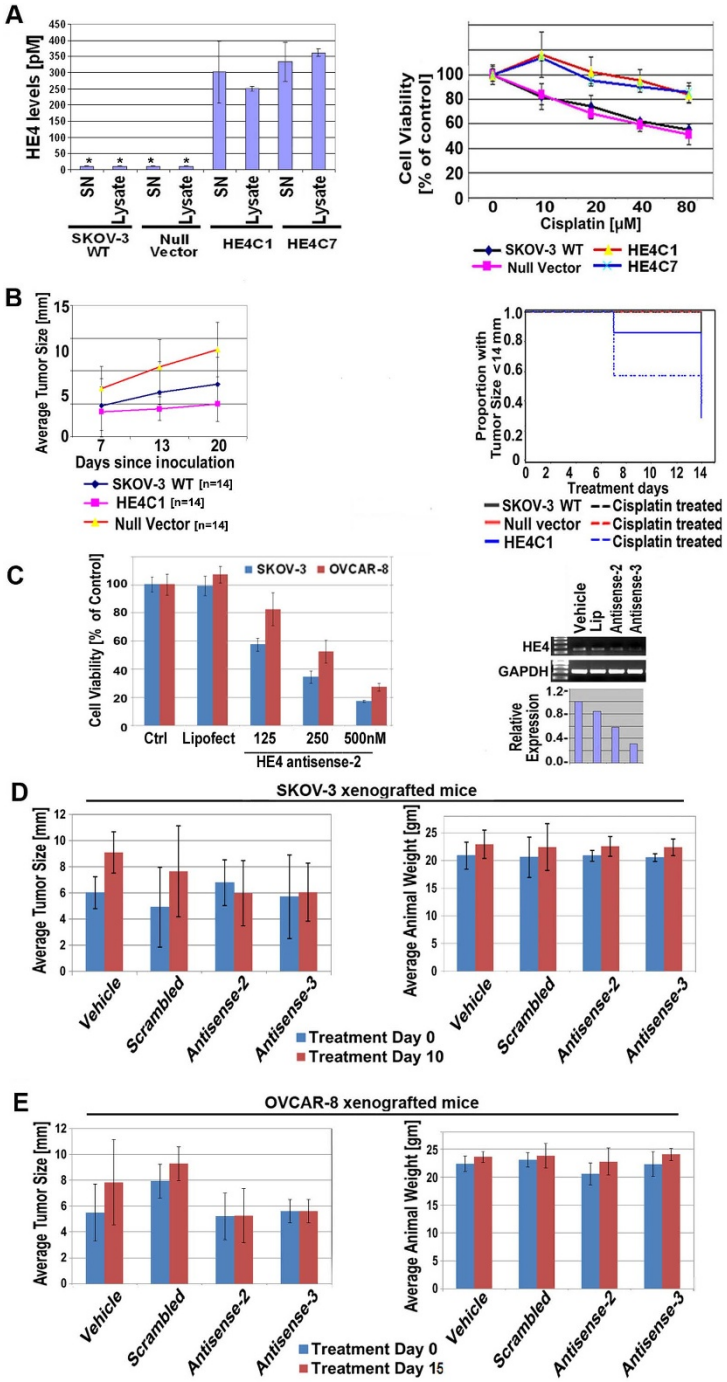


**Figure 2 | HE4 overexpression promotes tumor growth and chemoresistance against cisplatin.** (A) HE4 cellular (lysate) and media secretion (SN; supernatant, 24 h) levels in wild type SKOV-3, null vector clones and stably HE4 overexpression SKOV-3 clones (HE4C1, HE4C7) were determined by ELISA (left panel), * indicates HE4 values below 15pM. MTS based cell viability assay of WT SKOV-3, null vector and HE4 overexpressing clones treated with Cisplatin for 24 h revealed increased resistance by HE4 overexpressing cells. (right panel). (B) Tumor sizes of WT SKOV-3, null vector and SKOV-3 HE4C1 clone derived xenografts differed significantly during 20 days of trial (left panel). A Tukey-adjusted pairwise group difference showed that tumor size differed between SKOV-3 HE4C1 vs WT ($p=0.007$) and HE4C1 vs empty vector ($p<0.0001$) ($n=14$ each). There was no significant tumor size difference between the WT and null vector clone ($p=0.3$). Upon treatment with cisplatin for 14 days (days 20–34 from inoculation) cisplatin resistance and aggressive growth of HE4 overexpressing tumors was observed. Kaplan-Meier curve showed that HE4C1 derived tumors treated with cisplatin experienced excessive growth compared to cisplatin/vehicle treated WT SKOV-3 and null vector or HE4C1 treated with vehicle. Group difference among cisplatin treated groups was significant ($p=0.007$) and vehicle treated group showed $p=0.007$ (right panel). (C) MTS viability assay of SKOV-3 and OVCAR-8 cell-lines treated with novel HE4 antisense phosphoro-thio-oligonucleotides (PTO) for 24 hours (left panel). RT-PCR of SKOV-3 mRNA showed that Antisense-2 and Antisense-3 PTO reduced expression of HE4 (right panel) (D, E) Antisense-2 and Antisense-3 treatment (7 mg/kg, IP daily) stopped SKOV-3 (D, left panel; 10 day treatment) and OVCAR-8 (E, left panel; 15 day treatment) xenograft tumor growth in nude nice. PTO treatment) did not affect weight of animals xenografted with SKOV-3 (D, right panel) and OVCAR-8 (E, right panel).

lysate and Media showed that SKOV-3 HE4C1 produced average 302.6 pM and secreted 250.6pM of HE4. Similarly, HE4C7 produced average 333.6 pM and secreted 362.3pM of HE4 (Fig. 2A, left panel) and displayed increased HE4 mRNA expression (Supplementary Fig. 2A) compared with Null vector transformed and wild type SKOV-3 which had non-detectable HE4 levels (<15 pM). A MTS based in vitro cell viability assay demonstrated that HE4C1 and HE4C7 tumor cell lines were less sensitive to cisplatin (Fig. 2A, right panel) and paclitaxel treatment (Supplementary Fig. 2B) *in vitro* compared with controls. Similarly, HE4 overexpressing OVCAR-8 HE4C5 clones (Supplementary Fig. 2C) showed increased resistance to cisplatin (Supplementary Fig. 2D).

Next, we determined the effect of HE4 overexpression in ovarian cancer tumor growth *in vivo*. Mice xenografted with SKOV-3 HE4C1 showed aggressive tumor growth compared to the null vector and wild type SKOV-3 xenografted tumors (Fig. 2B, left panel). On day 20 the average tumor size in the HE4C1 group was 11 mm compared with 5–6 mm in the null vector and wild type SKOV-3 group. Similarly, Li et al. recently reported that HE4 overexpression promotes tumor growth in endometrial cancer cell lines and a xenograft mouse model[14]. Tukey-adjusted pairwise group difference analysis examining the tumor size between HE4C1, null vector and wild type derived tumors showed significantly larger tumor size for HE4C1 versus WT ($p=0.007$) as compared to the control groups versus null vector ($p<0.0001$). There was no significant difference in tumor size between the wild type and null vector clones ($p=0.3$). To determine a potential role of HE4 in the response to standard therapeutics, animals in each group were subdivided on day 20 after inoculation with tumor cells and treated with vehicle or cisplatin (10 mg/kg) for 14 days (Fig. 2B, middle and right panel). All seven wild type SKOV-3 xenografted animals (100%; 7/7) treated with vehicle and 86% of animals (6/7) that received cisplatin displayed tumors smaller than 14 mm on day 34. In the SKOV-3 null vector group tumors size stayed below 14 mm for all animals (100%; 7/7) when treated with vehicle or cisplatin. In contrast, examination of the HE4 overexpressing HE4C1 xenograft revealed that 5 of 7 (78%) animals treated with cisplatin had tumors that grew larger than 14 mm. Animals treated with vehicle in this group, only 2 of 7 (28%) had tumors greater than 14 mm. This indicates not only development of cisplatin resistance but enhanced cisplatin-induced tumor growth[15] of HE4 overexpressing cells as visualized by Kaplan-Meier analysis (Fig. 2B, right panel). There was a significant difference observed in tumor growth among cisplatin or vehicle treated SKOV-3 HE4C1 groups ($p=0.007$).

**Antisense targeting of HE4 suppressed ovarian cancer cell and tumor growth.** To further investigate HE4 as a therapeutic target for the treatment of ovarian cancer, HE4-antisense phosphorothioo-oligonucleotides (PTO) were synthesized (IDT Inc.; Coralville, IA) [antisense-2: 5′A*C*A*C*C*T*T* C*C*C*A*C*A*G*C*C*A* T*T3′; antisense-3: 5′G*A*C*A*C*C*T*T*C*C*C*A* C*A*G* C*C*A*T*T3′]. With the knowledge that SKOV-3 and OVCAR-8 ovarian cancer cell lines display cisplatin resistance and express base line levels of HE4, cell viability and tumor growth was evaluated after treatment with HE4-antisense PTO. HE4 antisense PTO reduced the cell viability of SKOV-3 and OVCAR-8 cells within 24 hours dose dependently (Fig. 2C, left panel). HE4 antisense PTO reduced the HE4 mRNA expression within 24 h (shown for SKOV-3; Fig. 2C, right panel). We evaluated the antitumor efficacy of antisense PTO in comparison to a scrambled PTO (5′C*T*C*A*G*G*A*T*G* G*C*G*G*A*G*C*G*G*T*C*T3′) in a subcutaneous xenograft model in nude mice. Within 10 days of daily (7 mg/kg, IP) administration, antisense-2 and antisense-3 PTO arrested the growth of SKOV-3 xenograft tumors relative to scrambled PTO or vehicle (Fig. 2D, left panel). Similarly, 15 day treatment with antisense-2 and −3 PTO (7 mg/kg, IP) decreased tumor growth in OVCAR-8 xenografted animals (Fig. 2E, left panel). Treatment with antisense-2 and −3 did not cause any adverse effect on animal weight during studies (Fig. 2 D/E, right panels).

**HE4 interaction with growth factor receptors and HIF1α.** To delineate HE4 mediated signaling interactions, we examined SKOV-3 xenograft tissues histochemically, as these tissues provide a snapshot of a tumor microenvironment where soluble factors, proteins, and host-tumor immune mediators converge to support tumor growth. Confocal microscopy revealed cytoplasmic as well as nuclear staining of HE4 in SKOV-3 xenograft tissues (Fig. 3A, left panel). In contrast, cultured ovarian cancer cells (SKOV-3, OVCAR-8; Fig. 3A middle panels) demonstrated only cytosolic staining. We screened the effect of recombinant growth factors on SKOV-3 (EGF, VEGF, Insulin), and OVCAR-8 (EGF, Insulin) cells to determine changes in levels or the cellular localization of HE4 (treatment for 30 min, see methods). The oncogenic role of these growth factors[16,17] in ovarian tumorigenesis is well established. Confocal microscopy revealed nuclear translocation of HE4 upon treatment with the growth factors listed above (Fig. 3A, middle panels). In addition, increased cellular expression of HE4 upon stimulation with EGF, Insulin or VEGF was measured (Fig. 3A, right panel). Remarkably, stimulation with insulin displayed intense nucleolar localization of HE4 in OVCAR-8 cells (Fig. 3A, lower middle panel). The stimulation with VEGF did not cause nuclear accumulation of HE4 in OVCAR-8 cells.

EGF-stimulated HE4 nuclear translocation suggested a potential interaction of HE4 with EGFR or other cell surface receptors. A confocal microscopy of SKOV-3 xenograft tissue revealed strong co-localization of HE4 and EGFR (Pearson Coefficient=~0.9) (Fig. 3B). Further, EGFR showed co-immunoprecipitation with HE4 (HE4 antibody as bait) as compared to IgG (Fig. 3C, left panel). Moreover, elevation in HE4 levels (e.g. in SKOV-3 HE4C1 and HE4C7 clones) elicited higher EGFR co-immunoprecipitation as compared to wild type SKOV-3 and a null vector clone. Measurement of intensity of HE4 expression after immuno staining was performed and revealed that treatment of cells with an EGF inhibitor (Iressa) inhibited EGF-induced HE4 overall staining in SKOV-3 cells. The measurements (relative intensity units/U) were as follows. No treatment: mean 615±130 U (45 fields); EGF treated: mean 909±225 U (40 fields); Iressa and EGF treated: mean 579±113 U (46 fields).

VEGF induced nuclear translocation of HE4 pointed to a potential correlation of HE4 expression with angiogenesis regulators such as HIF1α. SKOV-3 derived xenograft tissues showed strong co-localization of HIF1α and HE4 (Fig. 3D). Nuclear expression of HIF1α independently and in cohort with VEGF promotes aggressive and chemoresistant disease and denotes poor prognosis in ovarian cancer patients[18–20]. SKOV-3 xenograft tissue lysates showed co-immunoprecipitation of HIF1α with HE4 (Fig. 3E, left panel). Further, siRNA mediated specific HIF1α inhibition (Fig. 3E, middle panel) or treatment with HIF1α inhibitor 2-methoxy estradiol reduced HE4 expression concomitantly (Fig. 3E, right panel). Full length of the gels corresponding to Fig. 3 (middle and right are included in the supplementary section Fig. 3.

## Discussion

HE4 overexpression with serum levels >150 pm is common in 78% of ovarian cancer patients as compared to breast (13%), endometrial (25%), gastrointestinal (16%) and lung (42%) tumors[21]. High sensitivity and specificity of HE4 serum expression in EOC patients had led to the USFDA approval of HE4 as a biomarker for ovarian cancer. Similarly, the ROMA algorithm was approved to detect ovarian tumor among the women presenting with a pelvic mass or cyst[9,10].

The biological functions of HE4 despite recent implications in the immune defense[22,23] remain elusive. Previous reports attribute a role

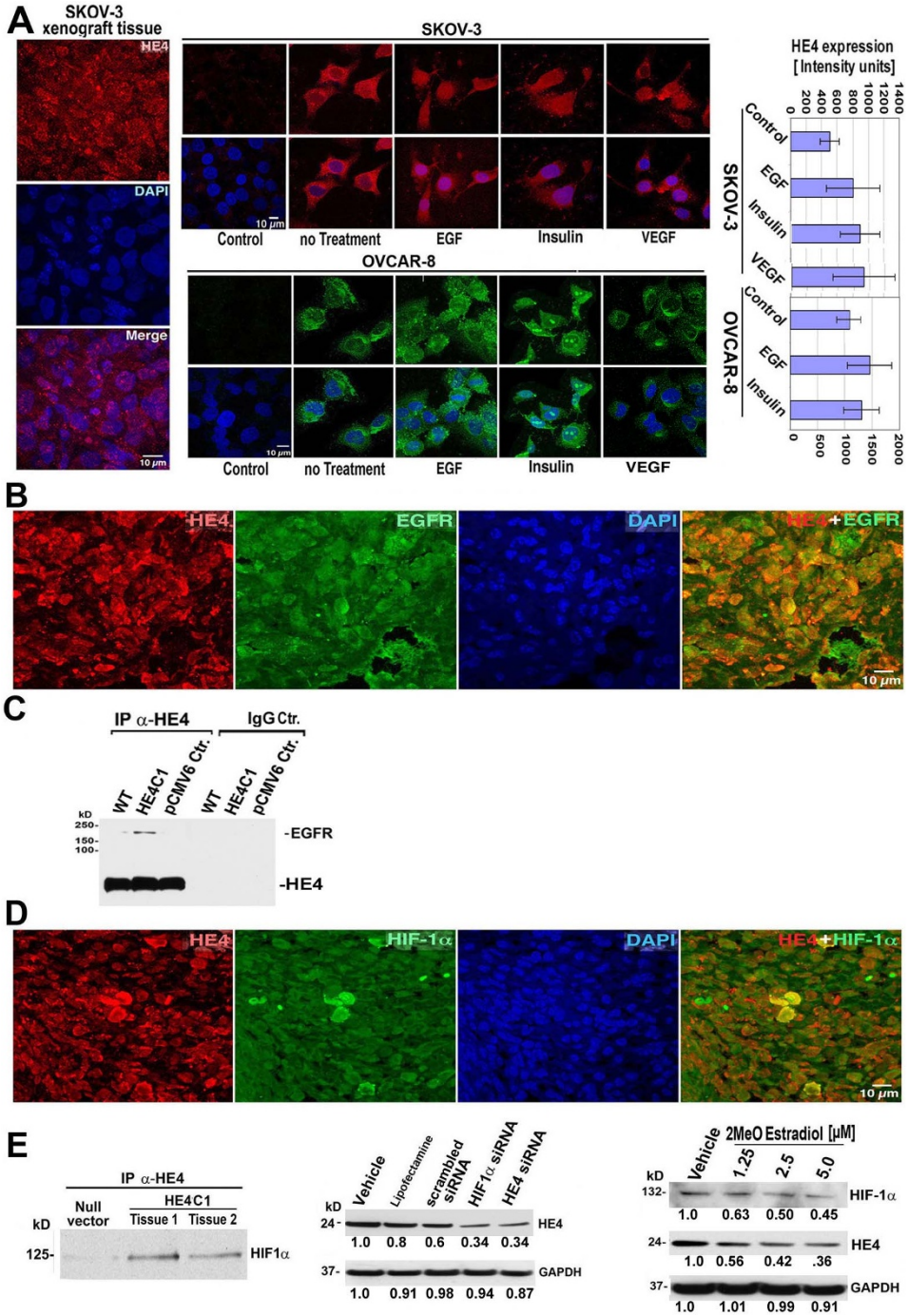


**Figure 3 | HE4 associates with EGFR and HIF1α.** (A) Confocal images of SKOV-3 xenograft tissue stained with HE4 primary antibody and corresponding Alexa Fluor secondary antibody and chromatin staining (DAPI). Intense nuclear HE4 staining was observed (left panel); Bar=10 μM. Confocal microscopy of serum starved SKOV-3 (upper middle panel) or OVCAR-8 (lower middle panel) cells upon stimulation with EGF (33 ng/ml), insulin (83 ng/ml) or VEGF (16.6 ng/ml) for 30 minutes showed intensified nuclear HE4 localization and increased overall expression of HE4 (right panel; average intensity of 6 fields is shown) as compared to non-treated cells. (B) Paraffin embedded SKOV-3 xenograft tumor tissues were processed and stained with antibodies against HE4 and EGFR and images analyzed by immunofluorescence microscopy. Yellow spots indicate co-localization of HE4 with EGFR. (C) HE4 was immunoprecipitated from the lysates of SKOV-3 WT cells and western blot analysis performed. EGFR co-immunoprecipitation was detected and increased in HE4 overexpressing HE4C1. (D) Paraffin embedded SKOV-3 derived xenograft tumor tissues were processed and stained with antibodies against HE4 and HIF1α and immunofluorescence microscopy carried out. Yellow spots indicate co-localization of HE4 with HIF1α. (E) Lysates of HE4C1 and null vector transformed SKOV-3 derived xenograft tumors were immunoprecipitated with HE4 primary antibody and probed for HIF1α by immunoblotting revealing HIF1α coprecipitation along with increased HE4 overexpression (left panel). HIF1α and HE4 siRNA treatment inhibited HE4 expression in SKOV-3 cells (middle panel). Lipofectamine and scrambled oligo were used as control. Treatment of SKOV-3 for 24 h with a HIF1α inhibitor (2MeOEstradiol) resulted in decreased HE4 expression (right panel).Gels were run in similar conditions. Full length gels corresponding to middle and right panel are shown in Supplementary Information section (Fig. 3, Supplementary Info).Only single bands were detected. Densitometric analysis of the immunoblots was carried out and the values relative to control (set to 1) are shown.

of HE4 expression in ovarian cancer cell line adhesion and motility[24] as well as in endometrial tumor growth in an animal model[14]. HE4 has also been shown to be a sensitive biomarker in renal fibrosis and inhibition of HE4 expression via a neutralizing antibody resolved kidney fibrosis in an animal model[25,26]. Our study demonstrates the impact of HE4 overexpression on ovarian cancer proliferation and on chemotherapy both *in vitro* and in an animal model. HE4 overexpressing cells derived from SKOV-3 and OVCAR-8 cell lines exhibited a diminished response to cisplatin and paclitaxel. In nude mice, xenografts derived from an HE4 overexpressing SKOV-3 clone formed larger tumors than the control groups during the 20 days of trial and subsequent treatment with cisplatin revealed development of drug resistance. These studies are concordant with serum HE4 levels and survival outcomes of 89 EOC patients that were examined for this study. We observed that patients with platinum resistant disease or HE4 overexpression both displayed lower survival compared to the platinum sensitive or low HE4 expressing groups ($p=0.001$). A previously published OVCAD study[27] and a study by Kang et al.[28] suggests a correlation of HE4 levels with platinum resistance and poor survival in ovarian cancer patients.

Our data identify HE4 as a selective molecular target to suppress ovarian cancer cell viability *in vitro* and tumor growth *in vivo* via PTO or alternative methods. Confocal microscopy revealed nuclear staining of HE4 in SKOV-3 xenograft tissues. Similarly, a previous study by Georgakopoulos et al.[29] suggested partial nuclear localization of HE4 in human ovarian tumor tissues. In contrast, cultured ovarian cancer cells demonstrated cytosolic staining. This observation led us to investigate plausible factors that may induce nuclear translocation of HE4. Our experiments reveal that the spatial expression of HE4 is linked to the activity of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and insulin, which induce nuclear or in the case of insulin also nucleolar translocation. Recent studies have suggested a role of the nucleolus in cancer progression and revealed oncogenes other than HE4 localizing to nucleoli such as p53, RB protein, c-Myc which target ribosomal biosynthesis in nucleoli[30–32]. EGF, VEGF and Insulin and their receptors are directly linked to ovarian tumor growth and chemoresistance[33–35]. The effect of VEGF on HE4 nuclear translocation suggested a role for HE4 in the angiogenic component of the tumor microenvironment. VEGF is essential for hypoxia-inducible factor-mediated neovascularization and regulated by the hypoxia-inducible factor (HIF) family[36]. Outcomes of immunoprecipitation assays, co-localization and application of HIF1α inhibitor 2-methoxyestradiol and siRNA revealed the interaction of HE4 with HIF1α. As for HIF1α, colocalization and coimmunoprecipitation of EGFR with HE4 supported interaction of HE4 with the cognate receptor of EGF.

Collectively, our data for the first time, implicate HE4 overexpression as a molecular driver for tumor growth and chemoresistance as reflected in decreased survival rates for ovarian cancer patients with tumors that overexpress HE4. We show evidence that HE4 expression and localization is correlated with the function of growth factors. Our study demonstrates that HE4 expression or interactions are potential targets for the treatment of ovarian cancer.

## Methods

**Cell culture.** Human cell lines SKOV-3 (ovarian adenocarcinoma), OVCAR-3 (ovarian epithelial adenocarcinoma), and OVCAR-8 (ovarian adenocarcinoma) were obtained from American Type Culture Collection (Manassas, VA). Cells were grown T75 cell culture flasks (Corning, New York, NY) in complete DMEM medium (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum and 1% pen/strep antibiotic according to the suppliers recommendation.

**Cell viability assay.** Viability of cells before and after drug treatment was determined was determined by the 96®Aqueous-One-Solution Assay (Promega, Madison, WI). Briefly, cells (5000/well) were plated into 96 well flat bottom plates (Corning, Inc., Corning, NY) before treatment with solution of cisplatin or paclitaxel in DMSO or vehicle (DMSO) as indicated. Following incubation at 37°C in a cell culture incubator for 20 h MTS reagent was added at a 1 : 10 dilution to the medium. The samples were incubated for an additional 4 h before absorbance was measured at 490 nm in an ELISA plate reader (Thermo Labsystems, Waltham, MA). Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations (X±SD) of a representative experiment in % of absorbance by samples with untreated cells (=100%).

**Development of HE4 overexpressing clones for in vitro and in vivo studies.** An HE4 overexpressing vector for stable expression was engineered by inserting the coding sequence of human WAP four-disulfide core domain 2 (WFDC2) cDNA into eukaryotic expression vector, pCMV6-entry (Origene, Rockville, MD). Transfection of the constructs in SKOV-3 and OVCAR-8 cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stably transfected cells were selected by resistance to G-418 (0.5 μg/ml) (Research Products International, Mount Prospect, IL), applied 48 h after transfection and continued during cell culture. G-418 resistant cells were seeded as 200 μl/well on a 96 well cell culture plate (Corning; New York, NY). Cells growing from single colonies (cloned; stable transfection) were isolated. Multiple clones were selected and cellular or secretory HE4 levels among clones were measured by ELISA (Fujirebio Inc; Philadelphia, PA) or PCR. SKOV-3, OVCAR-8 and the null vector or HE4 overexpressing clone xenograft experiments were performed as described previously[37].

**Stimulations and confocal microscopy experiments.** SKOV-3 and OVCAR-8 cells were grown to semi-confluence in complete DMEM medium in a lab-Tek 8 chamber slides. Cells were serum starved overnight and were stimulated with VEGF (16.6 ng/ml), EGF (33 ng/ml) or insulin (83 ng/ml) for 30 minutes. Cells were fixed with 10% neutral buffered formalin and washed with PBST two times. Fixed cells were incubated with rabbit HE4 primary antibody (HE4 rabbit mAb, cat no-TA307787, Origene, Austin TX;1 : 1000) overnight at 4°C, washed with PBST (3×5 min) and incubated with Alexa Fluor 488 or 546 secondary antibody (1 : 2000) for 2 hours at room temperature in the dark. Slides were washed (5×5 min) and DAPI containing mounting medium was applied. For co-localization studies paraffin embedded SKOV-3 derived xenograft tissues were processed and stained with primary HE4, EGFR (cat no-4405; Cell Signaling, Danvers, MA) or HIF1α (cat no-13515; Santa Cruz biotechnology, Santa Cruz, CA) antibody for 24 hours. Cells were washed and Alexa Flour 488 and 546 antibody was applied. Confocal images were obtained and processed as published earlier[37].

**Coimmunoprecipitation experiments.** SKOV-3, null vector and HE4 overexpressing clones HE4C1 and HE4C7 cells were seeded into 100 $mm^2$ dishes and cultured to ~80% confluency. Cells were rinsed in PBS, pH 7.4, scraped off in 1x lysis buffer (20 mM Tris-HCl, pH 7.5) 150 mM NaCl, 1 mM Sod-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Sodium Vanadate, 1 μg/ml Leupeptin, 1 mM PMSF. Lysates were rocked at 4°C for 5 min, sonicated (10 pulses, 5 sec), centrifuged at 14000 g for 10 min and protein concentration of the supernatant quantitated (BioRad Protein estimation kit, Hercules, CA). Lysates were adjusted to 1 μg/μl total protein concentration in the original lysis buffer and 200 μl lysates per sample used for each IP reaction. Identical samples were prepared for pulldown antibody (HE4, cat no-TA307787, Origene); EGFR (cat no-4405) or IGF1β receptor (cat no-6113, Cell Signaling, Danvers, MA) and Isotype IgG control (cat no- 3900 s, Cell Signaling) with Ab concentration identical at 40 ng/μl. 40 μl per sample of Protein G Sepharose (Invitrogen, Carlsbad, CA) 50% bead slurry were washed 3 times in 200 μl PBS and microcentrifuged at 10.000 rpm for 1 min). Beads were resuspended with Ab in PBS buffer, incubated at 4°C overnight and washed 3x in 400 μl PBS. Beads were added to each 200 μl lysate sample, incubated at 4°C on a rotator for 4 h, spun and washed 3x in 400 μl lysis buffer with 150 mM and 2x with 300 mM NaCl. The beads were resuspended in 20 μl Laemli buffer, vortexed, heated to 95°C for 3 min, spun and the supernatant loaded for PAGE analysis.


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## Author Contributions

R.K.S. and R.G.M. conceived and designed the studies. Patient studies were conducted by R.G.M. and S.M. Statistical analysis was performed by M.C.M. Clones were developed by N.Y., Y.D.T. and J.F.P. Animal experiments, *in vitro* experiments and cell stimulations were performed by E.K.H., T.H. and R.K.S. ELISA analyses were performed by G.L.M. Immunoprecipitations were performed by T.S.L., K.K.K. and N.Y. Manuscript was written by R.K.S., T.S.L. and R.G.M. Every author read and approved the manuscript.

## Additional information

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** R.G.M. and R.K.S. are listed as co-inventors on a pending patent application (US61/493881). Patent was assigned to Women and Infants Hospital of Rhode Island. Other authors declare that no competing interest exists.

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