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17β-Estradiol upregulates GREB1 and accelerates ovarian tumor progression *in vivo*

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Exogenous 17β-estradiol (E2) accelerates the progression of ovarian cancer in the transgenic tgCAG-LS-TAg mouse model of the disease. We hypothesized that E2 has direct effects on ovarian cancer cells and this study was designed to determine the molecular mechanisms by which E2 accelerates ovarian tumor progression. Mouse ovarian cancer ascites (MAS) cell lines were derived from tgCAG-LS-TAg mice. Following intraperitoneal engraftment of two MAS cell lines, MASC1 and MASE2, into SCID mice, exogenous E2 significantly decreased the survival time and increased the tumor burden. Microarray analysis performed on MASE2-derived tumors treated with E2 or placebo showed that E2 treatment caused the upregulation of 197 genes and the downregulation of 55 genes. The expression of gene regulated by estrogen in breast cancer 1 (*Greb1*) was upregulated in mouse tumors treated with E2 and was overexpressed in human ovarian cancers relative to human ovarian surface epithelium, suggesting a role for GREB1 in human ovarian tumor progression. RNA interference-mediated knockdown of GREB1 in MASE2 cells decreased their proliferation rate *in vitro* and increased survival time in mice engrafted with the cells. These results emphasize the importance of E2 in ovarian tumor progression and identify *Greb1* as a novel gene target for therapeutic intervention.

Cancer Genetics

Epithelial ovarian cancer (EOC) is the most lethal of the gynecologic malignancies.[1] The gravity of the disease is mainly attributed to advanced stage at diagnosis, with 79% of ovarian cancers detected after disease metastasis,[1] which includes the spread of cancer cells throughout the peritoneal cavity and the accumulation of abdominal ascites.[2] One of the risk factors for EOC is the use of exogenous steroid hormones after menopause, known as hormone replacement

Key words: ovarian cancer, estrogen, GREB1, mouse model, microarray

Abbreviations: α-MEM: alpha-minimum essential media; AdCre: adenovirus expressing Cre recombinase; AKT: protein kinase B; BCL2: B-cell lymphoma-2; DMEM/F12: Dulbecco's modified eagle medium: nutrient mixture F-12; E2: 17β-estradiol; EOC: epithelial ovarian cancer; ESR1: estrogen receptor alpha; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; GEO: gene expression omnibus; GFP: green fluorescent protein; GO: gene ontology; GOA: gene ontology annotation; GREB1: gene regulated by estrogen in breast cancer-1; HIF1α: hypoxia-induced factor 1α; HRT: hormone replacement therapy; IP: intraperitoneally; JNK: c-Jun N-terminal kinases; LMP: low melting point; MAS: mouse ascites (cell line); MASC: MAS cells derived from a control-treated mouse; MASE: MAS cells derived from an E2-treated mouse; MASP: MAS cells derived from a progesterone-treated mouse; MCF7: Michigan Cancer Foundation-7 (cell line); MGI: mouse genome informatics; MOSE: mouse ovarian surface epithelium; OSE: ovarian surface epithelium; OVX: ovariectomized; PPIA: peptidylprolyl isomerase A; Q-PCR: quantitative real-time RT-PCR; RMA: robust multi-array average; RT-PCR: reverse transcription polymerase chain reaction; SCID: severe combined immunodeficient; shGREB1: short hairpin corresponding to GREB1; SV40 Tag: Simian vacuolating virus 40 large tumor antigen; VEGF: vascular endothelial growth factor

Additional Supporting Information may be found in the online version of this article.

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What's new?

Post-menopausal estrogen-only hormone replacement therapy is associated with an increased risk of epithelial ovarian cancer, the development of which may be influenced by the actions of 17β-estradiol (E2) on ovarian surface epithelium (OSE). Here, in an orthotopic mouse model of ovarian cancer, E2 was found to accelerate tumor progression. Microarray analysis identified 197 E2-upregulated and 55 E2-downregulated genes. Among E2-upregulated genes was *Greb1*. GREB1 protein was highly upregulated in human tumors relative to normal human OSE, suggesting that it may be a tumor-promoting factor and potential mediator of E2-stimulated tumor growth.

therapy (HRT). Several epidemiologic studies have indicated that the increased risk of developing EOC is mainly associated with estrogen-only HRT[3,4] or current use of HRT.[5–7]

In addition to the epidemiologic evidence, several basic research studies have indicated that exogenous 17β-estradiol (E2) may increase the risk of developing EOC by acting directly on the ovarian surface epithelium (OSE), which is believed to be a tissue of origin for EOCs. OSE cells treated with E2 may be more susceptible to transformation due to the ability of E2 to increase proliferation,[8,9] produce oxidative DNA damage[10] and cause preneoplastic lesions in the OSE.[9,11] Although E2 can act on normal OSE cells to increase the risk of developing EOC, it can also accelerate EOC progression after tumor initiation. In tumorigenic human OSE cells, E2 inhibited apoptosis by upregulation of BCL2, and E2 has also been shown to reduce paclitaxel-induced apoptosis in the Caov-3 ovarian cancer cell line through alterations in the AKT/JNK signaling pathway.[12,13] We have previously shown that exogenous E2 accelerates ovarian cancer progression *in vivo*, resulting in a decrease in overall survival in a transgenic mouse model of ovarian cancer.[11] Additionally, it is known that the estrous cycle affects tumor growth in ovarian cancer xenografts, with increased tumor burden occurring in mice engrafted during the proestrous phase when E2 levels are the highest.[14] Similarly, E2 administration to ovariectomized mice inoculated with ovarian cancer cells significantly increased the tumor burden.[14]

The ability of E2 to enhance EOC progression may be mediated through changes in the tumor microenvironment, since E2 increased the expression of the extracellular matrix protein fibulin-1 in ovarian cancer cells.[15] An E2 metabolite, 4-hydroxy estradiol, induced hypoxia-inducible factor 1α (HIF1α) and vascular endothelial growth factor A (VEGFA) expression in ovarian cancer cell lines, suggesting that E2 can stimulate angiogenesis.[16] Moreover, adhesion of EOC cells to collagen *in vitro* is augmented by E2 treatment[14] and E2 enhances migration and induces epithelial–mesenchymal transition in EOC cells through upregulation of the transcription factors Snail and Slug, linking E2 to tumor invasion and metastasis.[17,18]

Although the biological consequences of E2 on EOC etiology are diverse, the molecular mechanisms underlying these effects are not well characterized. Breast cancer research has identified some E2-inducible gene targets involved in tumor progression, including gene regulated by estrogen in breast cancer 1 (*GREB1*).[19] *GREB1* was first identified as a hormone-responsive gene in the breast cancer cell line MCF7.[19] Although the function of GREB1 is still unknown, studies have indicated that it is an early response gene that is a key regulator of E2-stimulated breast cancer cell growth.[20] Recently, GREB1 was identified as the top estrogen receptor alpha (ESR1)-interacting protein in MCF7 cells treated with estrogen.[21] Moreover, it is an essential co-factor for ESR1 mediated transcription in breast cancer cells.[21] GREB1 expression in EOC has not been investigated, despite EOC being well-established as a hormone responsive cancer.

Previously, we demonstrated that exogenous E2 accelerates ovarian tumor initiation in a transgenic mouse model of ovarian cancer, tgCAG-LS-TAg mice, by causing putative preneoplastic lesions in the OSE and inducing an earlier onset of tumorigenesis, resulting in a decreased overall survival.[11] In the present study, we aimed to investigate the effects of E2 on ovarian cancer progression. Using mouse ascites (MAS) cell lines derived from the hormone-responsive tgCAG-LS-TAg model of ovarian cancer, we tested the ability of E2 to increase cell growth *in vitro* and *in vivo*. Although E2 treatment had little effect on *in vitro* MAS cell proliferation, it significantly accelerated tumor progression of MAS cell grafts *in vivo*. This discrepancy suggests that E2 is promoting tumor progression through mechanisms other than proliferation. E2 has been shown to decrease apoptosis in human ovarian cancer cells[12,13] and upregulate angiogenesis-related factors.[16] *Greb1* emerged as an E2-regulated molecular target in our microarray and was expressed in both mouse and human ovarian cancers. Knockdown of *Greb1* in the MASE2 cell line decreased the proliferation rate *in vitro* and increased the survival time of mice grafted with the cells *in vivo*. These results highlight the importance of E2 in EOC progression and identify GREB1 as a novel mediator of this process.

Material and Methods

Generation of mouse ascites cell lines and cell culture

MAS cells were obtained from a previously described transgenic mouse model of ovarian cancer.[11] The tgCAG-LS-TAg model utilizes the Cre-LoxP system to inducibly express the SV40 large and small T-Antigens (SV40 TAg) in the OSE cells following the intrabursal injection of adenovirus expressing Cre recombinase, as previously described.[11,22] At the time of necropsy, ascites cells from the tgCAG-LS-TAg mice were

collected *via* needle aspiration of the abdominal ascites. The MAS cells were grown in MOSE media as previously described.[23] Six polyclonal cell lines were derived and PCR analysis confirmed that the cell lines expressed SV40 TAg, indicating that the cell lines were derived from tgCAG-LS-TAg mouse ovarian cancer cells. MASC1 and MASC2 were obtained from the ascites of tgCAG-LS-TAg mice treated with a placebo pellet (as previously described in Ref. 11, MASE1 and MASE2 were established from the ascites of mice treated with E2, and MASP1 and MASP2 were derived from mice treated with progesterone (P4). All cell lines were cultured in the absence of steroid hormones, unless otherwise indicated. A2780cp (Dr. M. Molepo, Ottawa, ON), HEY (Dr. G. Mills, Houston, TX) and ES-2 cells (Dr. J. Bell, Ottawa, ON) were grown in phenol-red free Dulbecco's modified eagle medium (DMEM)/F12 + 5% serum. OVCAR-3 (ATCC) were grown in RPMI + 20% serum, and OVCA 432 (Dr. G. Mills, Houston, TX) were grown in αMEM + 10% serum. Cells were routinely tested for Mycoplasma and were authenticated by short-tandem repeat profiling in April 2013.

Short hairpin GREB1 knockdown

Lentiviral particles encoding a short hairpin directed against *Greb1* (shGREB1) were obtained from Open Biosystems (Thermo Scientific, Ottawa, Canada). The sequence (TGCTGTTGACAGTGAGCGCTCGCTTCAGTGTCATGAAG AATAGTGAAGCCACAGATGTATTCTTCATGACACTGAA GCGATTGCCTACTGCCTCGGA) corresponded to both isoforms of mouse *Greb1* as well as isoform A of human *GREB1*. Particles encoding a non-silencing construct were used as a control. Both shGREB1 and non-silencing constructs included a green fluorescent protein (GFP) tag from the GIPZ vector. MASE2 cells were infected with lentiviral particles and selected in puromycin (2 μg/mL). Two shGREB1 constructs were tested and the one with the most efficient knockdown was used in all subsequent experiments.

Cell proliferation assays

MAS cell lines were seeded at 15,000 cells per well in 6-well dishes (Corning Inc., Corning, NY) and 24 hr after plating were treated with either 500 nM of E2 (Sigma-Aldrich, St. Louis, MO) or the vehicle control. After 48 hr of E2 treatment, the cells were counted using a Coulter Counter (Beckman Coulter, Mississauga, ON, Canada). For the hormone treatments, charcoal purification was used to eliminate any residual steroid hormones present in the fetal bovine serum (FBS). Charcoal (50 mg/mL) was added to the FBS for 24 hr and then the FBS was centrifuged at 1800*g* for 30 min. The supernatant was collected and centrifuged at 27,000*g* for 60 min. MASE2 cells expressing either a non-silencing construct or shGREB1 were seeded at 50,000 cells per well in 6-well dishes in α-MEM plus 10% FBS. When cells were attached (3–4 hr after plating), media was changed to α-MEM plus 1% FBS. Viable cell counts were measured with a ViCell XR Cell Viability Analyzer (Invitrogen, Burlington, Canada) at 0, 24, 48, 72 and 96 hr after changing to 1% FBS.

Soft-agar colony formation assay

MASC1 and MASE2 cells were used in this assay along with the human ovarian cancer cell line A2780cp (which served as a positive control) and mouse OSE cells (negative control), which were collected as described previously.[23] Two concentrations of UltraPure low melting point (LMP) soft agar (Invitrogen, Burlington, Canada) were prepared and sterilized by autoclaving—a base layer of 2% LMP agarose and a top layer of 1% LMP agarose which were mixed with equal volumes of 2× (DMEM)/F12 media (phenol red free, Sigma-Aldrich, St. Louis, MO) containing 5% charcoal purified FBS and either E2 (250 nM) or vehicle control. Cells were added to the top layer soft agar (1%) and after two weeks, two dilutions (8 wells) per cell line were counted and expressed as a percentage of the input cells.

MAS cell grafts into immunodeficient SCID mice

MAS cells (10^7 cells/animal) were injected intraperitoneally (IP) and tumor progression assessed in three experiments. First, MASC1 and MASE2 cells were injected IP into 5–6 weeks old Fox Chase SCID mice (CB17-*Prkdc*^{scid}/NCrCrl, Charles Rivers Laboratories, Montreal, QC). Five days after injection, a 60-day slow release hormone pellet (Innovative Research of America, Sarasota, FL) was surgically implanted subcutaneously in the neck region. Mice were treated with either E2 (0.25 mg/pellet) or a placebo pellet (N = 7–9 mice per group). In the second experiment, ovariectomized (OVX, 8–9 weeks old) female Fox Chase SCID mice were grafted with either MASC2 or MASE2 cells and either an E2 (0.25 mg/pellet) or a placebo pellet was inserted subcutaneously and concomitantly with the IP injection of cells (N = 5–7 mice per group). The third experiment assessed the effects of GREB1 knockdown by injecting MASE2 non-silencing shRNA or MASE2 shGREB1 cells IP into 6–8 week old female Fox Chase SCID mice (N = 8 mice per group). For all experiments, mice were housed in sterile conditions on a 12L:12D cycle, with free access to food and water and were euthanized when they reached a loss-of-wellness endpoint. The survival time is the amount of time from the day the cells were injected until the day when the mice reached a loss-of-wellness endpoint including: weight loss > 15% of body weight, body weight increase > 5g with respect to age-matched controls, presence of abdominal distension that impairs mobility, respiratory distress, dehydration and/or anorexia. All animals were euthanized *via* CO_2 asphyxiation. All animal experiments were performed according to the *Guide to the Care and Use of Experimental Animals* established by the Canadian Council on Animal Care, with protocols approved by the University of Ottawa Animal Care Committee.

Preparation and analysis of tissues

Tissues were collected from mice at necropsy, and the volume of ascites and wet weight of the total tumor burden

were recorded. Tissues were either flash frozen or fixed in 10% neutral-buffered formalin overnight, transferred to 70% ethanol and paraffin-embedded. Flash-frozen tumor samples were lysed and protein was extracted using the ProteoJet Mammalian Cell Lysis Reagent according to the manufacturer's instructions (Fermentas, Thermo Scientific, Ottawa, Canada, K0301). GREB1 (Sigma-Aldrich, St. Louis, MO, HPA024616) and GAPDH (Abcam, Toronto, Canada, #ab8245) antibodies were used for Western blotting. For histological analyses, 3–5 μm sections were cut and stained with hematoxylin and eosin (H&E). Tissue sections were scanned and imaged using the Aperio ScanScope and the Aperio ImageScope program (Aperio Technologies, Inc., Vista, CA). Tumor proliferation was determined by performing immunohistochemistry on the tumors with the proliferation marker Ki67 (1:25, DAKO, Burlington, Canada) as previously described.[11] Positive pixel counting was performed to detect Ki67 positive nuclei using the Aperio ImageScope program. Four random fields of view (×200 magnification) were counted and averaged to obtain a mean for each tumor. At least four tumors per group (MASC1 + placebo, MASC1 + E2, MASE2 + placebo and MASE2 + E2) were stained and assessed.

Microarray analysis

Three biological replicates (MASE2 tumors grown in SCID mice) were analyzed for both conditions (addition of E2 or placebo) on Affymetrix microarray platform Mouse Gene 1.0 ST Array that measures the expression of approximately 28,850 genes. Signal intensities were normalized and summarized with variance stabilization normalization[24] and RMA,[25] respectively, and probe sets differentially expressed between both conditions (E2 and placebo) were detected with limma.[26] Gene functions were analyzed with Gene Ontology (GO) terms annotations from the MGI and GOA for mouse and human, respectively, and orthologs between both species mapped by NCBI's Homologene.[27] Pathway annotations were obtained from the Kyoto Encyclopedia of Genes and Genomes.[28] Microarray data are publically accessible from the GEO database at record GSE45271.

Human ovarian cancer tissue samples

Samples of primary tumor tissue were acquired from the Ottawa Ovarian Cancer Tissue Bank under a protocol approved by The Ottawa Hospital Research Ethics Board. The tumor samples were obtained with informed consent from patients with serous, endometrioid and clear cell histological subtypes.

RNA extraction and quantitative RT-PCR

RNA was extracted from MASE2 tumors, MAS cell lines, primary human OSE cell cultures (C19(P5), C14(P5) and C26(P4) cells at early passage), human ovarian cancer cell lines and primary human ovarian tumor tissue using the RNeasy kit (Qiagen, Toronto, Canada) according to the manufacturer's instructions. cDNA was prepared using the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Burlington, Canada) according to the manufacturer's instructions. Quantitative RT-PCR (Q-PCR) was performed on mouse tissue using cDNA (100 ng) and primers for *Esr1, Esr2, Greb1, Cyp11a1, Stc2* and *Pgr* relative to mouse whole ovary cDNA, with 18S or *Ppia* used as an endogenous control. For the human tissues, *GAPDH* or *PPIA* was used as the endogenous control and expression of *GREB1* was presented relative to MCF7 cDNA (positive control). Primer details are contained in Supporting Information Table 1. Q-PCR reactions were set up in 96-well plates and run on the Applied Biosystems 7500 Fast RT-PCR system using SYBR green fast reaction settings (ABI, Carlsbad, CA). Data were analyzed using the ABI 7500 software (version 2.0.1).

Statistical analyses

All data were plotted using GraphPad Prism software (GraphPad Software, San Diego, CA). MAS cell proliferation *in vitro, Esr1, Esr2*, and *Greb1* expression *in vitro* and Ki67 proliferation in tumors were compared using a One-Way ANOVA with a Tukey's *post hoc* test. Colony formation was compared using a *t* test. Kaplan–Meier survival curves were compared using a Log-rank test. Mouse tumor weights, ascites volumes and sites of dissemination were compared using *t* tests. Q-PCR validations of *Greb1, Cyp11a1, Stc2* and *Pgr* were compared using a *t* test. Cellular proliferation rates were compared using linear regression analysis. Significance was inferred at $p < 0.05$.

Results

E2 treatment does not affect the growth of mouse ascites cells *in vitro*

MAS cell lines were derived from the peritoneal ascites of tgCAG-LS-TAg mice with ovarian tumors. Each of the MAS cell lines originated from an individual mouse that was treated *in vivo* with either placebo (MASC1 and MASC2 cell lines), P4 (MASP1and MASP2 cell lines) or E2 (MASE1 and MASE2 cell lines). The MAS cell lines grew at comparable rates *in vitro* (in the absence of hormone treatments) and treatment with E2 (500 nM for 48 hr) did not affect the proliferation of any of the cell lines compared to the vehicle controls (Fig. 1*a*). Moreover, a range of E2 doses (from 1 nM to 1000 nM) did not affect the proliferation of any of the MAS cell lines after 24, 72 or 96 hr compared to controls (data not shown). Soft agar colony formation assays were used to determine if E2 enhanced anchorage-independent growth of MAS cells. The MASC1 and MASE2 cells formed colonies with an efficiency of approximately 1% after 2 weeks (Fig. 1*b*, inset). E2 treatment (250 nM) did not affect the ability of the MAS cells to form colonies. The human ovarian cancer cell line, A2780cp, was used as a positive control for the assay and these cells produced colonies with 80–90% efficiency (Fig. 1*b*). Only 0.1% of the mouse OSE cells, used as a negative control, formed colonies (Fig. 1*b*, inset). Neither

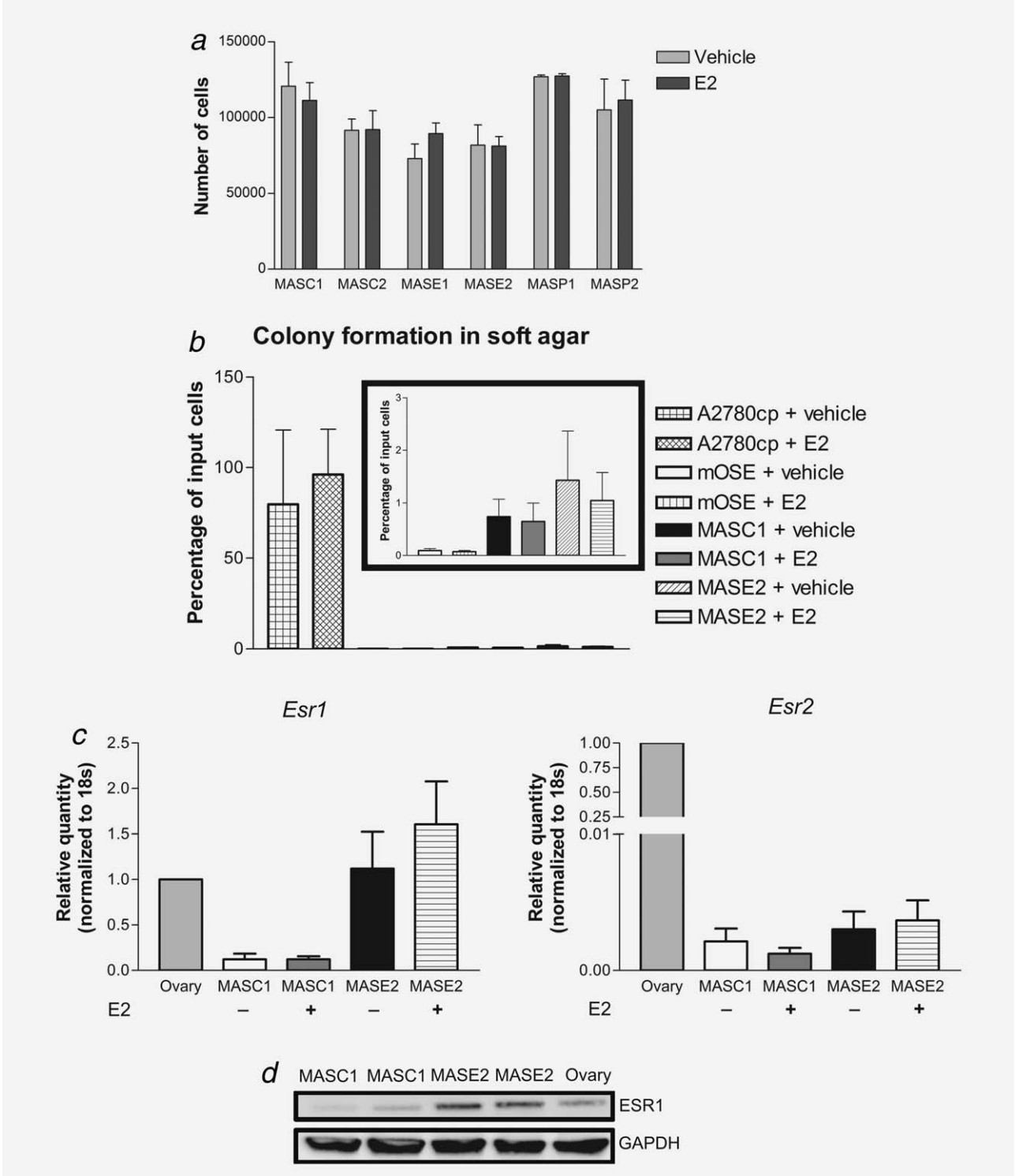

Figure 1. E2 treatment did not affect *in vitro* proliferation or substrate-independent growth of mouse ovarian cancer cells. (*a*) The MAS cell lines proliferated at comparable rates and treatment with 500 nM of E2 did not alter the rate of proliferation over 48 hr (compared to those treated with vehicle). (*b*) E2 (250 nM) did not affect the ability of MASC1 and MASE2 cells to form colonies in soft agar ($n = 3$; inset is a close up of the mOSE, MASC1 and MASE2 data). (*c*) Q-PCR analysis revealed that MASE2 cells had higher endogenous *Esr1* mRNA levels than MASC1 cells ($n = 3$, $p < 0.05$, One-Way ANOVA and Tukey *post hoc* test), but the abundance of *Esr1* transcripts was not altered by treatment with E2 (500 nM). *Esr2* transcript levels were very low in the MAS cell lines and were not induced by E2 treatment. (*d*) ESR1 protein expression (in duplicate) was higher in the MASE2 cells compared to the mouse ovary (control) and to the very low levels seen in the MASC1 cells.

A2780cp nor mouse OSE cells produced more or fewer colonies in response to E2 treatment (Fig. 1*b*).

Because the MAS cells did not show enhanced proliferation in response to E2 treatment *in vitro*, Q-PCR was used to determine whether the estrogen receptors alpha (*Esr1)* and beta (*Esr2)* were expressed in the cultured cells. *Esr1* was expressed in the MASE2 cell line at a level comparable to the positive control, whole mouse ovary, and the level of expression was not enhanced by E2 (500 nM for 48 hr *in vitro*; Fig. 1*c*). Similarly, *Esr1* expression was not increased by E2 in the MASC1 cell line. *Esr2* mRNA expression was very low in both the MASC1 and MASE2 cell lines compared to mouse ovary and was not increased by E2 treatment (Fig. 1*c*). In agreement with the mRNA data, Western blot analysis demonstrated that the MAS line derived from a mouse treated with E2 *in vivo* (MASE2) expressed higher levels of endogenous ESR1 protein than those derived from a placebo-treated control mouse (MASC1; Fig. 1*d*).

E2 treatment accelerates tumor progression *in vivo*

In order to determine the effects of E2 treatment on tumor growth *in vivo*, two of the MAS cell lines, MASC1 and MASE2, were orthotopically injected IP into SCID mice, with subsequent insertion of a placebo or E2 slow release pellet. For both cell lines, E2 treatment significantly accelerated the progression of tumorigenesis (Fig. 2*a*). SCID mice grafted with MASC1 cells and treated with E2 had a significantly shorter median survival time compared to the placebo controls (42 *vs.* 56 days; Fig. 2*a*; $p < 0.0001$, Log-rank test, $N = 9$ mice/group). There was a trend toward an increase in tumor burden ($p = 0.07$, *t* test, Fig. 2*a*), with the mean total tumor weight at disease endpoint in mice receiving a placebo pellet being 1.88 ± 0.46 g compared to 2.99 ± 0.34 g in mice treated with E2. Ascites volume was significantly increased in mice treated with E2 compared to controls ($p = 0.007$, *t* test; Fig. 2*a*). The majority (63%) of control mice injected with MASC1 cells developed ascites with a mean volume of 0.85 ± 0.37 mL, while 100% of the E2 treated mice had peritoneal ascites with a mean volume of 2.02 ± 0.14 mL (Fig. 2*a*).

Similarly, SCID mice grafted with MASE2 cells and treated with E2 reached endpoint significantly faster than the placebo controls (30.5 *vs.* 68 days; Fig. 2*a*; $p = 0.0004$, Log-rank test, $N = 7$–8 mice/group). E2 treatment significantly increased the tumor weights in mice grafted with MASE2 cells with a mean total tumor weight of 5.51 ± 0.74 g compared with 1.63 ± 0.14 g in placebo-treated controls ($p < 0.0001$, *t* test). In contrast to the MASC1 cells, there was a trend toward a decrease in the incidence and volume of ascites in mice treated with E2 (Fig. 2*a*). The mean volume of ascites was 2.2 ± 0.74 mL (75% of mice) in the placebo-treated mice and only 0.24 ± 0.19 mL (40% of the mice) in mice given E2 (Fig. 2*a*). Similar E2-induced decreases in survival were obtained when OVX SCID mice were grafted with the MASC2 or MASE2 cells (Supporting Information Fig. 1).

Histological examination of the tumors from the SCID mice revealed aggressive, poorly differentiated carcinomas with a high mitotic grade (Fig. 2*b*). Areas of necrosis were apparent in the tumors derived from both cell lines (Fig. 2*b*, low magnification, arrows). Papillary structures, reminiscent of the serous histological subtype of EOC, were seen at the exterior edge of the tumors from all of the groups (Fig. 2*b*, high magnification images). Immunohistochemistry for the proliferation marker Ki67 was performed on tumors collected at disease endpoint, when the carcinomas should be at the most aggressive stage. E2 did not affect the fraction of proliferating MAS cells *in vivo* (Fig. 2*c*), as determined by the percentage of positive pixels (cells expressing Ki67) in tumors from at least four mice per group (One-Way ANOVA and Tukey's post-test; Fig. 2*c*).

E2 increases expression of genes involved in cell differentiation, proliferation and migration

The molecular mechanisms by which E2 accelerates ovarian cancer progression were investigated by performing gene expression profiling on tumors from mice treated with either placebo or E2 pellets. Microarray analysis was performed on mouse tumors derived from the MASE2 orthotopic model, because this cell line exhibited the greatest *in vivo* growth response to E2 (Fig. 2*a*). E2 treatment resulted in a statistically significant upregulation of 197 genes and a downregulation of 55 genes (Supporting Information Table 2). Gene Ontology analysis revealed that many of the upregulated genes belonged to pathways involved in cell differentiation, cell proliferation, cell migration, angiogenesis and the steroid metabolic process, such as *Greb1, Pgr, Cyp11a1, Cdh2, Fgf7, Ednra, Kdr* and *Bmp6* (Table 1). GREB1 protein levels in both MASC2 and MASE2 tumors were increased by E2 treatment *in vivo* (Fig. 2*d*). MASE2 tumors treated with or without E2 obtained from a separate experiment (using OVX mice; Supporting Information Fig. 1) were used to validate a subset of the microarray targets by Q-PCR. *Greb1* and *Cyp11a1* mRNA levels were significantly elevated and there was a trend toward increased levels of *Pgr* and *Stc2* in tumors treated with E2 compared to the placebo controls (Fig. 3). The gene expression levels in the tumors were expressed relative to the levels in whole mouse ovary, which was used as a positive control. Both *Pgr* and *Stc2* were elevated in E2-treated tumors relative to normal ovary.

GREB1 is expressed in human and mouse ovarian cancers and can be induced in cells that express ESR1

Greb1 was one of the most highly induced genes after E2 treatment of mouse ovarian tumors. To determine whether *GREB1* might also be expressed in human ovarian cancers, transcript levels were determined in tumors of four histological subtypes of EOC: high grade serous, low grade serous, endometrioid and clear cell ($N = 4$–5 per subtype). *GREB1* expression was higher in every tumor sample compared to cultures of normal human OSE cells. The average *GREB1*

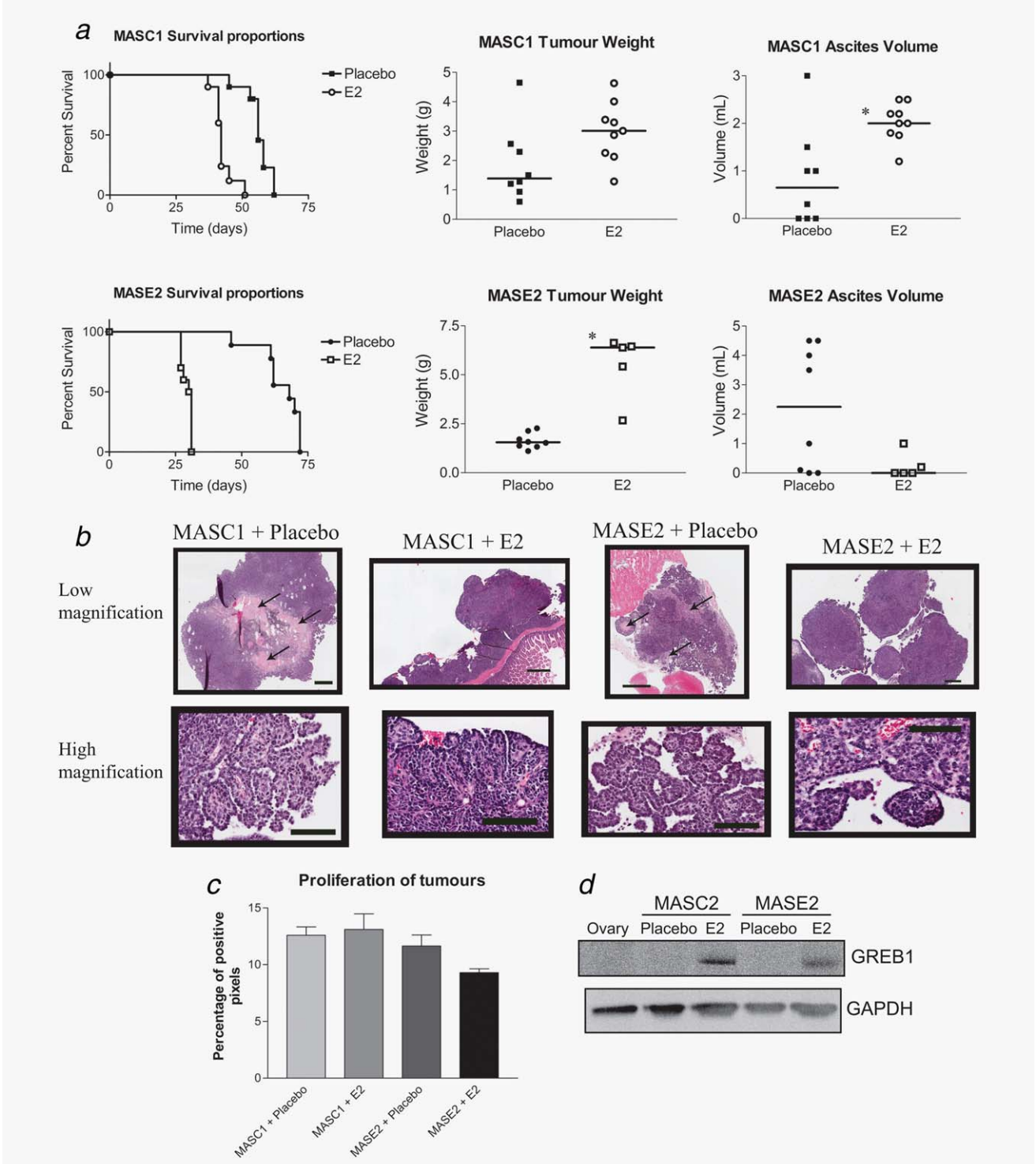

Figure 2. Exogenous E2 treatment decreased the survival time of immunodeficient mice grafted IP with mouse ovarian cancer cell lines MASC1 and MASE2. (*a*) MASC1 or MASE2 cells were injected IP into SCID mice and 5 days later the mice were treated with E2 or placebo (*via* a subcutaneous pellet). Mice treated with E2 had a significantly shorter survival time compared to those treated with placebo (MASC1 survival curve, $p < 0.0001$, $n = 9$ mice/group; MASE2 survival curve $p = 0.0004$, $N = 7–8$ mice/group, Log-rank test). Mice grafted with MASC1 cells and treated with E2 had significantly elevated volumes of ascites compared to placebo controls (*$p = 0.0070$, *t* test, $N = 8–9$). The tumor burden of mice grafted with the MASE2 cells and treated with E2 was significantly more than the placebo controls (*$p < 0.0001$ *t* test, $N = 5–8$). (*b*) The tumors from MASC1 and MASE2 cells grafted into SCID mice were highly mitotic tumors with serous papillary structures. Low magnification scale bars are 500 μm and the high magnification scale bars are 100 μm. (*c*) E2 treatment did not affect the proliferation (Ki67 immunoreactivity) of ovarian cancer cells *in vivo* (One-way ANOVA, $N = 4–5$ tumors/group). (*d*) E2 treatment *in vivo* induced GREB1 expression in both MASC2 and MASE2 tumors.

Table 1. Genes differentially expressed in mouse ovarian tumors treated with E2 (compared to placebo)

Gene		Gene ontology pathway	log2FC	Adjusted *p* value
Upregulated by E2				
Cyp11a1	Cytochrome P450 (scc)	Steroid metabolic process	4.26	0.0006
Greb1	Gene regulated by estrogen in breast cancer protein	Unknown	3.41	0.0015
Fgf7	Fibroblast growth factor 7	Cell proliferation	2.65	0.0044
		Cell division		
Pgr	Progesterone receptor	Reproduction	2.64	0.0446
		Transcription		
		Cell proliferation		
		Cell differentiation		
Cdh2	N-cadherin	Cell differentiation	2.18	0.0044
		Cell migration		
Ednra	Endothelin receptor type A	Cell differentiation	2.11	0.0281
		Angiogenesis		
Kdr	Vascular endothelial growth factor receptor 2	Cell proliferation	2.07	0.0170
		Cell migration		
		Cell differentiation		
		Angiogenesis		
Taf4b	TAF4B RNA polymerase II, TATA box binding protein (TBP)-associated factor	Transcription	1.96	0.0156
Stc2	Stanniocalcin 2	Unknown	1.90	0.0201
Bmp6	Bone morphogenetic protein 6	Cell differentiation	1.31	0.0375
		Inflammatory response		
Downregulated by E2				
Sgpp1	Sphingosine-1-phosphate phosphatase 1	Apoptosis	-0.85	0.0281
Met	Met proto-oncogene	Cell differentiation	-1.18	0.0442
		Cell migration		
		Cell proliferation		
Ptgs1	Cyclooxygenase-1	Cell proliferation	-1.47	0.0315

Abbreviations: log2FC, logarithmic 2 of.

expression was 347 times higher in tumors than in OSE, and is similar to the level expressed by the positive control cell line MCF7 (303-fold increase, Fig. 4*a*). The level of *GREB1* mRNA expression was variable among the tumors and the histological subtypes, but the average fold increase per histological subtype relative to OSE cells is as follows: 93 times in high grade serous ($N = 5$), 310 times in low grade serous ($N = 4$), 93 times in clear cell ($N = 4$) and 297 times in endometrioid ($N = 5$). *GREB1* mRNA was present at low levels in several human ovarian cancer cell lines, HEY, ES-2 and A2780cp (Fig. 4*b*). Each of these cell lines also exhibited very low to undetectable levels of *ESR1* mRNA (Fig. 4*c*) and *in vitro* treatment of these cells with E2 did not increase expression of *ESR1* or *GREB1* (Figs. 4*b* and 4*c*). In contrast, two ovarian cancer cell lines known to express *ESR1* did show an E2-stimulated increase in *GREB1* mRNA (Fig. 4*d*). *Greb1* was also detected in the MAS cell lines MASC1 and MASE2 (Fig. 4*e*) and MASE2 cells treated with E2 (500 nM) exhibited a greater than 50-fold increase in *Greb1* mRNA, compared to the vehicle controls ($p < 0.05$, One-way ANOVA). An increase in GREB1 protein expression in the MASE2 cells treated with E2 corresponded with the increase in mRNA levels (Figs. 4*e* and 4*f*). MASC1 cells had very low levels of *ESR1* (Fig. 1*c*), and *Greb1* expression was not altered by E2 treatment (Fig. 4*e*) and was undetectable by Western blot analysis (data not shown).

GREB1 knockdown prolongs survival time in an orthotopic model of ovarian cancer

Stable GREB1 knockdown was achieved in the MASE2 cells using shRNA encoded in a GIPZ lentiviral vector. Following puromycin selection, there was >50% *Greb1* knockdown in shGREB1 cells relative to cells infected with a non-silencing construct; however, a different shGREB1 construct (shRNA2

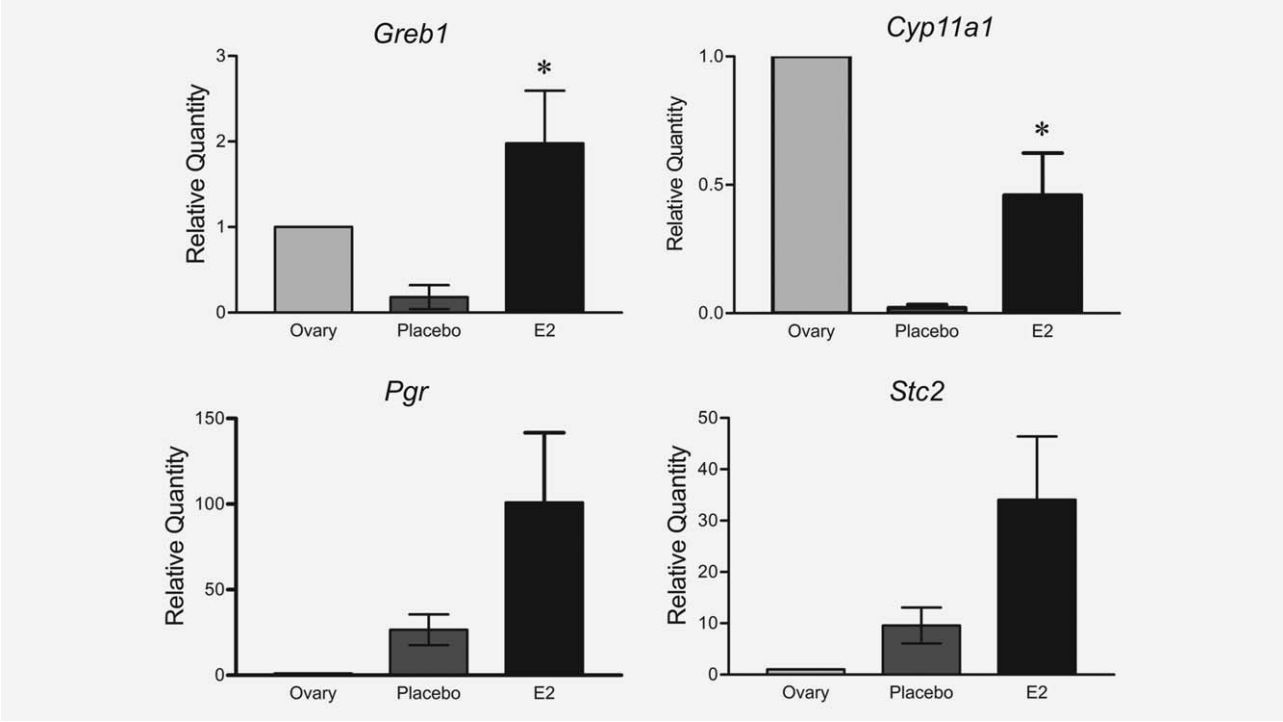

Figure 3. Q-PCR validation of four microarray targets in MASE2-derived tumors ($n = 4–5$ tumors/group) treated with and without E2. *Greb1* (*$p = 0.02$, *t* test) and *Cyp11a1* (*$p = 0.02$, *t* test) gene expression was significantly increased in tumors from mice treated with E2 compared to controls. There was a strong trend toward increased mRNA expression of *Stc2* ($p = 0.07$, *t* test) and *Pgr* ($p = 0.09$, *t* test) in E2-treated tumors compared to placebo controls. Whole mouse ovary was used as a positive control.

GREB1) did not decrease *Greb1* mRNA expression (Fig. 5*a*). GREB1 knockdown significantly decreased the proliferation rate of MASE2 cells *in vitro* over the course of 72 hr (Fig. 5*b*; $p = 0.021$, linear regression). We repeated these experiments with a separate transduction of MASE2 cells and found a similar decrease in proliferation in cells expressing the same construct (shRNA3, Supporting Information Fig. 2D). The shRNA3 GREB1 cells grew more slowly in all replicates than the control (non-silencing shRNA) cells, but there was too much variability between replicates for statistical significance by linear regression (after 72 hr, one-sample *t*-test, $p = 0.0215$). Knockdown was confirmed by Q-PCR (data not shown) and Western blot analysis (Supporting Information Figs. 2A and 2B).

To examine the role of GREB1 in ovarian cancer progression, MASE2 cells were injected IP into SCID mice. GREB1 knockdown (shGREB1) slowed the progression of ovarian cancer, reduced metastasis and significantly prolonged the survival of the mice (median 74 *vs.* 40.5 days, $N = 8$ mice/group; Fig. 5*c*; $p < 0.0001$, Log-rank test). The MASE2 cells infected with the non-silencing control shRNA formed GFP positive tumors throughout the peritoneal cavity, in tissues such as the diaphragm and intestines (Figs. 5*d* and 5*e*). In contrast, knockdown of GREB1 in the MASE2 cells reduced their ability to disseminate and form tumors throughout the peritoneum; very few metastases were observed in the mice injected with the shGREB1-infected cells (Figs. 5*d* and 5*e*).

Discussion

In this study, we set out to determine the effects of E2 on ovarian cancer cell growth and to elucidate the molecular mechanisms by which E2 modulates ovarian cancer progression. Using cell lines established from the peritoneal ascites (MAS cell lines) of our previously described transgenic model of ovarian cancer, tgCAG-LS-TAg mice, we have shown that E2 is able to accelerate ovarian cancer progression *in vivo* despite little effect on *in vitro* proliferation of the MAS cell lines. We used immunodeficient mice for this study because we have observed previously that syngeneic mice injected with T-antigen-expressing cells do not form tumors, due to the immunogenicity of T-antigen (unpublished results). Although this limits our ability to study the role of the immune system, our results indicate that E2 promotes tumor progression even in immunocompromised animals, suggesting that the immune system is not a major mediator of E2 action.

Gene expression analysis of E2 treated ovarian tumors indicated that E2 affects a host of genes involved in cell proliferation, differentiation and angiogenesis. Importantly, we have identified an E2 responsive gene, *Greb1*, which has not previously been shown to affect ovarian cancer progression. GREB1 enhances the hormone-stimulated growth of breast and prostate cancers, but has not been investigated in ovarian carcinomas. Herein we demonstrated that *Greb1* is induced by E2 in mouse ovarian tumors and is highly expressed in a sampling of four histological subtypes of human EOC. *GREB1* is upregulated by E2 in two

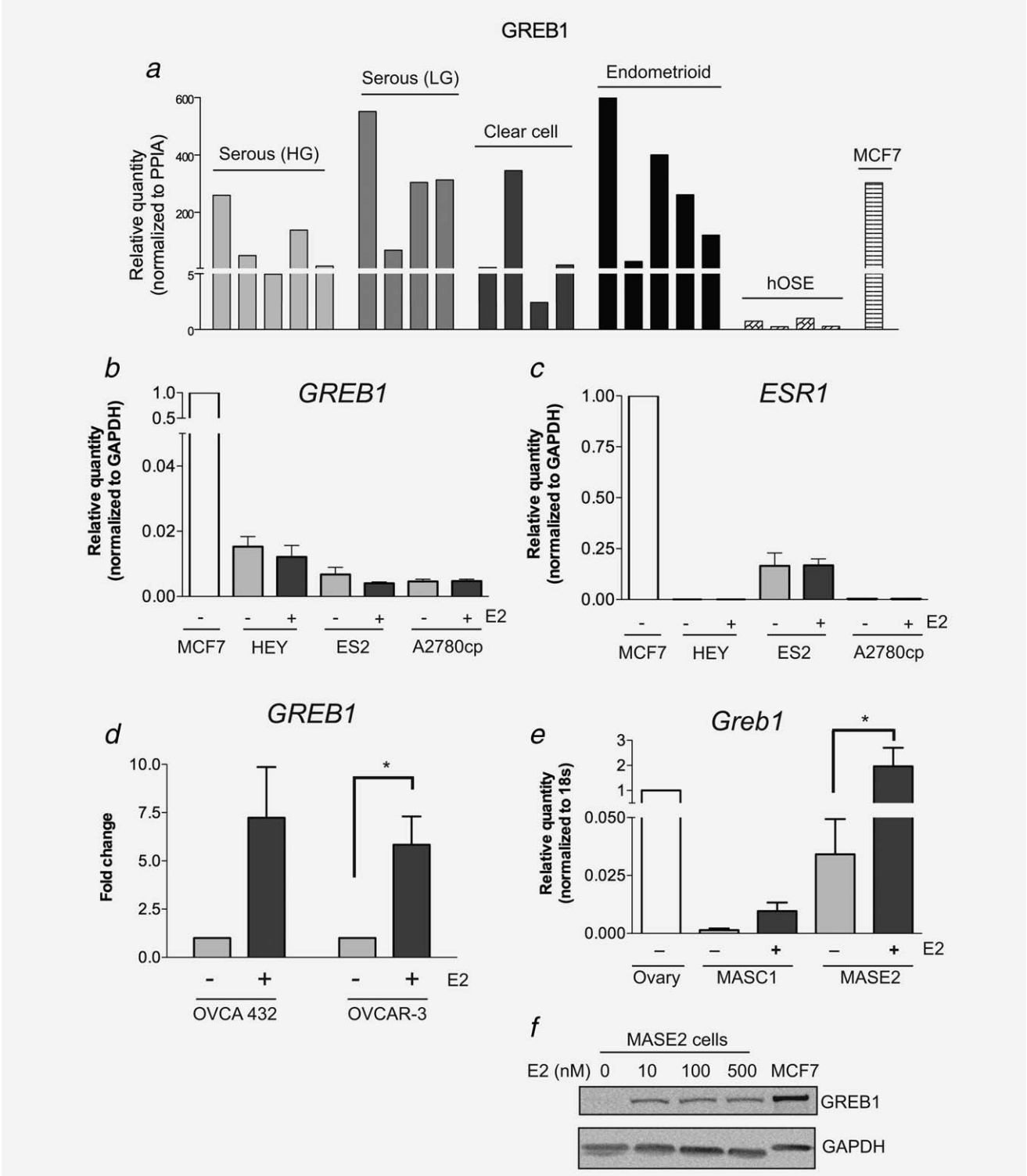

Figure 4. *Greb1* mRNA was expressed in human and mouse ovarian cancers. (*a*) *GREB1* was highly expressed in four epithelial ovarian carcinoma subtypes, including high grade serous, low grade serous, endometrioid and clear cell ($N = 4–5$ tumors/subtype), but was almost absent in four independent early-passage cultures of human OSE cells. MCF7 breast cancer cells were used as a positive control for *GREB1* mRNA expression. (*b*) *GREB1* was expressed in the human ovarian cancer cell lines HEY, ES2 and A2780cp, but was not induced by E2 treatment *in vitro*. (*c*) *ESR1* mRNA levels were very low in ES2 cells and were undetectable by Q-PCR in HEY and A2780cp cells. (*d*) *GREB1* levels were upregulated by E2 in two ESR1-expressing ovarian cancer cell lines, OVCA 432 ($p = 0.051$) and OVCAR-3 cells ($*p = 0.016$). (*e*) *Greb1* mRNA was present in MASC1 and MASE2 cells and was significantly increased in the MASE2 cells following treatment with 500 nM E2 for 48 hr *in vitro* ($*p < 0.05$). (*f*) GREB1 protein level was increased in MASE2 cells by *in vitro* treatment with E2 (10–500 nM). GAPDH was used as a loading control.

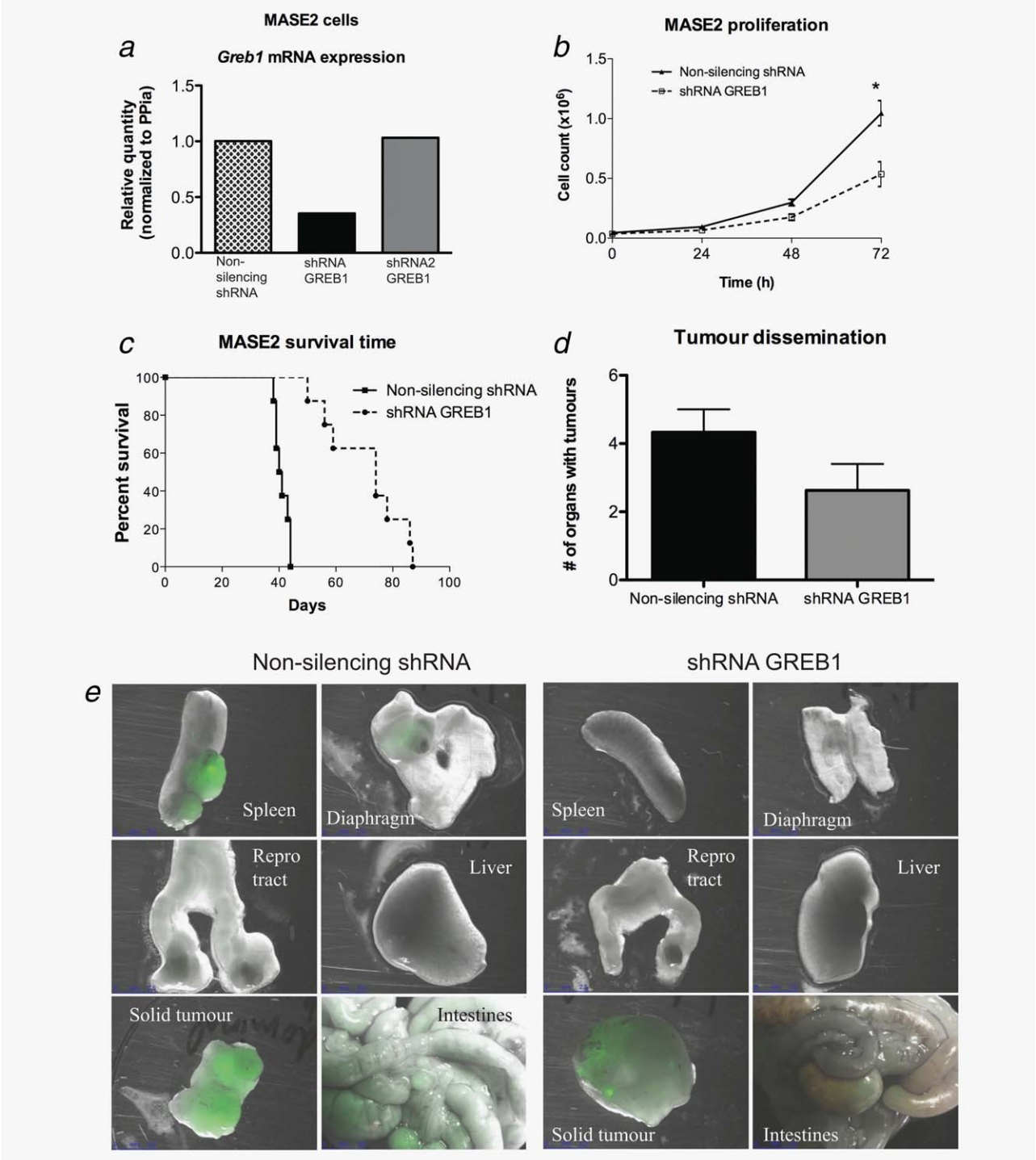

Figure 5. GREB1 knockdown slows the proliferation of MASE2 cells and prolongs survival in an orthotopic model of ovarian cancer. (*a*) Q-PCR demonstrating decreased mRNA expression of *Greb1* in MASE2 cells following shRNA knockdown with construct 1 (shRNA GREB1) but no knockdown with construct 2 (shRNA2 GREB1). (*b*) GREB1 knockdown in the MASE2 cells significantly slowed cellular proliferation (*$p = 0.021$, linear regression). (*c*) Knockdown of GREB1 (shRNA GREB1) significantly prolonged survival (median survival 74 days) after grafting to SCID mice (non-silencing shRNA controls, median survival time 40.5 days, $p < 0.0001$, Log-rank test). (*d*) GREB1 knockdown decreased the dissemination of tumors throughout the peritoneal cavity (non-silencing shRNA, $N = 6$; shRNA GREB1, $N = 8$). (*e*) The reduction in tumor dissemination was visualized by immunofluorescent microscopy (green immunofluorescence indicates the presence of cancer cells). Repro, reproductive

ESR1 expressing human ovarian cancer cell lines but not in cell lines expressing little or no *ESR1*, suggesting that *ESR1* is required for E2 induction of *GREB1* (as previously shown in breast cancer cells).[19] Knockdown of *Greb1* in ovarian cancer cells slows their proliferation rate *in vitro* and extends survival in an orthotopic ovarian cancer model, at least in part, by

decreasing the ability of the cells to disseminate and grow on abdominal tissues. GREB1 therefore appears to be a novel mediator of ovarian cancer dissemination and progression.

E2 treatment of the MAS cells did not affect *in vitro* proliferation, but significantly increased their progression as ovarian cancer *in vivo*. The limited effect of E2 on MAS cell proliferation *in vitro* may be due to low expression levels of the estrogen receptors in most cultured cells compared to the high level of expression of ESR1 (as evidenced by immunohistochemical staining) in the original tgCAG-LS-TAg ovarian tumours.[11] Although *Esr1* mRNA was detected in both the MASC1 and MASE2 cells *in vitro*, the expression was much higher in the MASE2 cell line and only the MASE2 cell line upregulated GREB1 when treated *in vitro* with E2. The MASE2 cell line also responded more robustly to E2 *in vivo* (compared to the MASC1 cell line which expressed very little ESR1). However, both cell lines developed into tumors that upregulated GREB1 in response to E2 treatment *in vivo*, and both showed a decrease in survival with E2 treatment, suggesting that both cell lines have sufficient ESR1 expression to respond to E2 *in vivo*. In addition to E2's established role as a mitogen, previous studies have shown that E2 aids in cell survival and the ability of OSE and ovarian cancer cells to evade apoptosis.[13,29] Mechanistically, this effect may be due to the ability of E2 to upregulate BCL2, a key anti-apoptotic protein.[12] In combination, these observations suggest that the effects of E2 on cell survival is more evident during *in vivo* tumor growth, where cell survival may be more challenging (as seen by the areas of tumor necrosis) compared to cell culture conditions.

To elucidate novel genes and signaling pathways regulated by E2 during *in vivo* ovarian cancer progression, we performed a microarray study on tumors obtained from our orthotopic model of ovarian cancer treated with E2. Interestingly, the array analysis revealed that *Greb1* is upregulated by E2 in mouse ovarian cancers. *GREB1* expression has previously been linked to breast and prostate cancer growth,[20,30] but has never been examined in ovarian cancer. Although the function of GREB1 is not known, the gene is induced following ESR1 binding to three estrogen response elements located upstream of the transcription start site.[31,32] Expression of *GREB1* is increased by exogenous E2 in breast cancer cells and following treatment with androgens in prostate cancer cells.[19,20,30–32] Suppression of *GREB1 via* RNA interference inhibited the hormone-stimulated proliferation of both breast and prostate cancer cells.[20,30] We found that *Greb1* is upregulated in mouse tumors and ascites cell lines treated with E2 and is more highly expressed in a small set of human EOC representing the major histological subtypes (serous, endometrioid and clear cell), relative to human OSE cells. Knocking down GREB1 with shRNA slowed the proliferation rate of the mouse ovarian cancer cells *in vitro* and decreased their dissemination *in vivo*, thus prolonging overall survival in an orthotopic mouse model. Although the mechanism by which GREB1 regulates proliferation remains unknown, a recent study indicates that, in breast cancers, GREB1 acts as a chromatin-bound ESR1 co-activator and stabilizes interactions between ESR1 and other co-factors, thus enhancing ESR1-mediated transcription.[21] Our results suggest that GREB1 is a novel mediator of ovarian cancer dissemination and progression, may have value as a therapeutic target and should be investigated for its potential to predict response to endocrine therapies in women suffering from EOC.

In addition to *Greb1*, the microarray study identified other genes that were induced by exogenous E2 during ovarian cancer progression, such as *Stc2, Pgr* and *Cyp11a1*. These three genes are known to be E2-responsive,[33–38] but their ability to affect ovarian tumourigenesis has not been examined. STC2 is a glycoprotein hormone that is expressed in the ovary and is induced by E2 in ESR1 positive breast cancer cells.[33–35,39,40] PGR is expressed in normal ovary and malignant EOCs.[41–44] *Cyp11a1* is found in the ovary and encodes for an enzyme responsible for the first step in the steroid hormone biosynthesis pathway, the conversion of cholesterol to pregnenolone.[45,46] Each of these gene targets may be important mediators of hormone-regulated EOC initiation and progression and warrant further characterization.

A previous study designed to examine the transcriptional profiles of human OSE and EOC cells treated with E2 *in vitro* revealed gene expression changes in pathways such as signal transduction, protein modification, apoptosis, protein biosynthesis, transcription, cell cycle progression, differentiation and cell adhesion.[47] The study focused on five E2-regulated genes, *RNPS1, ADD1, rap-2, SKIIP* and *PLXNA3*, that were differentially expressed in cultured EOC cells compared to non-malignant OSE cells.[47] Although expression of these five genes was not altered in our study, there was consistency between the two gene array studies in some of the pathways impacted by E2 in ovarian cancer, such as cell differentiation, transcription, apoptosis and cell adhesion. This would suggest that while similar pathways are affected, different genes might be regulated by E2 during *in vivo* tumor growth compared to *in vitro* cell growth. Another array study linked E2-regulated changes in protein expression in human ovarian tumors to a predictive response of these patients to an aromatase inhibitor.[48] Although that study examined a small number of proteins, there was some overlap with those identified in our gene expression study, including *KRT7*.[48] These results suggest that the mouse models described in our study could be used for testing novel therapeutics and for the preclinical identification of genes that predict a response to endocrine therapy.

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Author Contributions

LAL, KMH and BCV conceived and designed the study, developed methodology, analyzed data and wrote the manuscript. Experiments were performed by LAL, KMH and NM. Microarray analysis was performed by CPI.

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