Original Research

Under-expression of the opioid growth factor receptor promotes progression of human ovarian cancer

Renee N Donahue, Patricia J McLaughlin and Ian S Zagon

Department of Neural and Behavioral Sciences, The Pennsylvania State University, College of Medicine, Hershey, PA 17033–0850, USA
Corresponding author: Dr Ian S Zagon, Department of Neural and Behavioral Sciences, H109, The Milton S Hershey Medical Center, 500 University Drive, Room C3729, Hershey, PA 17033, USA. Email: isz1@psu.edu

Abstract

The opioid growth factor (OGF) and its receptor, OGFr, serve as a tonically active inhibitory axis regulating the proliferation of human ovarian cancer cells. In the present study, we have investigated the repercussion on the progression of this deadly neoplasia when cells are engineered to molecularly under-express OGFr. shRNA constructs were used to knockdown OGFr in SKOV-3 cells; two clonal cell lines were examined. OGFr protein expression was decreased up to 73% in clones compared with wild-type (WT) and empty vector (EV) controls. OGFr-binding assays of clones revealed 50–55% decreases in binding capacity compared with control cells; binding affinity was comparable in all groups. Cell number in clones was increased 33–132%, and doubling times decreased 29–35%, compared with WT and EV cultures. Addition of exogenous OGF or naltrexone did not affect cell number in cultures with silenced OGFr. DNA synthesis of clonal cell lines was increased 136–146% from the WT and EV groups; no changes were noted in cell survival. Nude mice injected subcutaneously with cells under-expressing OGFr had an increased tumor incidence, decreased latency to tumor formation, increased tumor volume and decreased OGFr expression in tumors compared with WT and EV controls. OGF treatment in mice with WT or EV tumors, but not OGFr under-expressing tumors, inhibited tumor volume and weight. Collectively, these data demonstrate the critical nature of the OGF–OGFr axis as a determinant of the progression of human ovarian cancer, and suggest that attenuation of this system has an important bearing on the survival of these patients.

Keywords: cell proliferation, [Met^5]-enkephalin, opioid, opioid growth factor receptor, OGFr, ovarian cancer, enkephalin

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Introduction

With an estimated 225,000 new cases of ovarian cancer worldwide reported each year, resulting in an estimated 140,200 deaths annually,[1] this neoplasia is the leading cause of death from gynecological malignancies.[2] Approximately 90% of primary ovarian cancers are epithelial in origin,[3] and the majority (75%) of cases are diagnosed in advanced stages (FIGO stage III/IV).[4] Despite 75–80% of women initially responding to standard treatments (cytoreductive surgery and adjuvant chemotherapy), more than 65% relapse within 12–18 months of therapy and, thereafter, all subsequent treatments are palliative.[5] A more profound understanding of the complex biology of this disease represents the most promising avenue to improve the prognosis of ovarian cancer patients.[4]

An integral component of the ovarian cancer phenotype is dysregulation of cell proliferation.[6] One native biological regulator of cell replication in normal cells and a wide variety of cancers, including ovarian cancer, is the opioid growth factor (OGF) and its receptor, OGFr.[7–12] Chemically termed as [Met^5]-enkephalin, OGF is a constitutively active native opioid peptide that is autocrine produced and secreted, and interacts with OGFr to delay the G_1/S interface of the cell cycle by upregulating cyclin-dependent kinase inhibitory pathways[12–15] without affecting cell survival.[12,16] Although OGFr has pharmacological characteristics of classical opioid receptors (recognizes opioids, naloxone reversibility and stereospecificity), this receptor shares no homology with classical opioid receptor nucleotide or amino acid sequences, and has a different cellular localization.[7,17–20] Regulation of cell proliferation by the OGF–OGFr axis involves nucleocytoplasmic trafficking from the outer nuclear envelope to the nucleus that requires nuclear localization signals and transport by karyopherin β and Ran.[18,21] OGF has been detected by radioimmunoassay in surgical samples taken from human neoplasms of the ovary,[22] and OGFr RNA and protein expression, as well as binding activity, have been documented in human

ovarian cancer cells.[12] Using an *in vitro* model, Donahue *et al.*[12] have identified OGF as the only opioid peptide inhibiting human ovarian cancer cell proliferation, and OGFr was established as the opioid receptor interacting with OGF to maintain the pace of cell replication. Attenuation of OGF–OGFr interfacing by continuous exposure to the opioid antagonist naltrexone (NTX), neutralization of OGF by antibodies to the peptide, or transient knockdown of OGFr using siRNA, resulted in an increase in ovarian cancer cell number relative to control levels.[12,23] Furthermore, the addition of OGF in ovarian cancer cultures having a transient knockdown of OGFr did not depress cell proliferation.[12,23] Treatment of intraperitoneal xenografts of human ovarian cancer cells in nude mice with OGF markedly suppressed tumor burden (tumor nodule number and tumor weight) through a mechanism that depresses cell proliferation and angiogenesis but does not alter cell survival.[24] Finally, treatment of mice with established tumors of human ovarian cancer cells with a combination of OGF and either taxol or cisplatin reduced tumor volumes and weight more than either agent alone.[25]

Stable overexpression of the OGFr in human ovarian cancer cells downregulates cell proliferation *in vitro* and inhibits tumorigenesis in mice.[26] In the present report, we have asked the question as to the repercussions of having a stable under-expression of OGFr in human ovarian cancer cells. We found that persistent molecular under-expression of OGFr increased cell proliferation *in vitro* and the development of ovarian tumors when cells were subcutaneously inoculated into nude mice. Moreover, in contrast to the inhibition in progression of neoplasia seen with OGF treatment in mice having normal OGFr concentrations in their tumors, mice with xenografts under-expressing OGFr did not respond to exogenous OGF. These results demonstrate the important role that the OGF–OGFr axis plays regarding the onset and progression of human ovarian cancer.

Materials and methods

Cell culture

The human ovarian cancer cell line SKOV-3[27] was obtained from the American Type Culture Collection (Manassas, VA, USA), and grown in a humidified atmosphere of 5% CO_2/95% air at 37°C in RPMI medium supplemented with 1.2% sodium bicarbonate, 10% fetal calf serum, 5000 U/mL penicillin, 5 μg/mL streptomycin and 10 mg/mL neomycin.

Transfection and clonal selection

Synthetic double-stranded oligonucleotides derived from OGFr siRNA (5′ GATCCGGTCGAGGTGTTTAAAAGCTT CAAGAGAGCTTTTAAACACCTCGACCTGA 3′) (Penn State University College of Medicine Core Facility) were cloned into a pSilencer 4.1–CMV hygro (Ambion, Austin, TX, USA) expression vector (OGFr shRNA). SKOV-3 cells were transfected for 24 h in antibiotic-free media with OGFr shRNA and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to generate clones. Four hours after initiation of transfection, cells were supplemented with serum containing medium. The negative control pSilencer 4.1–CMV hygro vector (Ambion), with limited homology to human coding cDNAs, was used to generate empty vector (EV) clones. Transfected cells were selected by growth in media containing hygromycin-B (50,000 mU/mL; EMD Chemicals, Gibbstown, NJ, USA); two clones (SH-6, SH-16) were expanded and maintained in media containing hygromycin-B. Untransfected wild-type (WT) and EV cells served as controls.

Cell growth

Cells were plated and counted 24 h later (time 0) to determine seeding efficiency. For treatment studies, 10^{-6} mol/L OGF or NTX (Sigma-Aldrich, Saint Louis, MO, USA) was added at time 0; media and compounds were replaced daily. Compounds were prepared in sterile water and dilutions represent final concentrations. An equivalent volume of sterile water was added to control wells. Cells were harvested at designated times, stained with trypan blue and counted with a hemacytometer. At least two aliquots/well from two wells/treatment/timepoint were sampled.

Animals

Four week-old athymic nu/nu female mice, purchased from Charles River Laboratory (Wilmington, MA, USA), were housed in pathogen-free isolator ventilated cages in a controlled-temperature room (22–25°C) with a 12–12 h light/dark cycle (lights on 07:00–19:00). Sterile rodent diet (Harlan Teklad, Frederick, MD, USA) and water were available *ad libitum*. All procedures were approved by the IACUC committee of the Pennsylvania State University, College of Medicine, Hershey, PA, USA, and conformed to the guidelines established by the National Institute of Health. Mice were allowed 48 h to acclimate before experimentation.

Tumor cell implantation and treatment

For preliminary tumor burden studies, three concentrations (1×10^6, 2×10^6 or 4×10^6) of clonal cell lines stably under-expressing OGFr (SH-6 and SH-16), as well as EV and WT cells, were injected subcutaneously into the right scapula region (~0.1 mL/mouse) of three unanesthetized mice/group. In the subsequent treatment study, using 8–12 mice/group, animals receiving a single concentration (1×10^6) of WT, EV or SH-6 cells were randomly assigned to receive daily intraperitoneal injections of OGF (10 mg/kg) or an equivalent volume of saline. Treatments were initiated when tumors were first observed. OGF was dissolved in saline and prepared weekly.

Tumor growth

The day of tumor cell inoculation was considered day 0. Mice were weighed weekly and observed daily, and the date that a tumor became 'visible' was recorded.

Individual tumors were measured with vernier calipers (accuracy $\pm$ 0.05 mm) every three days. Measurements of the largest perpendicular dimensions were recorded, and tumor volume was calculated using the formula: $l \times w^2 \times \pi/6$ where length (l) is the longest dimension and width (w) is perpendicular to the length.[28] The time a tumor was considered measurable was the day when tumor volume was 5 mm or greater in diameter ($\sim$62.5 mm^3).

Termination day measurements

Mice were euthanized by an overdose of sodium pentobarbital (100 mg/kg) and cervical dislocation at 21 d following tumor cell inoculation. Upon sacrifice, mice were weighed, tumors and spleens removed and weighed, and the lymph nodes, liver and spleen examined for metastases. Tumors were processed for immunohistochemistry for OGF and OGFr, and Western blotting for OGFr concentrations.

Semi-quantitative immunohistochemistry

Immunohistochemistry was utilized to evaluate the presence and relative levels of OGF and OGFr in cells and tumor tissue following published procedures.[12] Cells were grown on coverglasses for 72 h, while tumors were excised, frozen in chilled isopentane and sectioned at 10 μm; preparations were fixed, permeabilized and stained with antibodies to OGF and OGFr that were generated in our laboratory.[29] To evaluate relative expression levels, images were taken at the same exposure time. In cells, the mean intensity of staining was determined for at least 100 cells/group from three coverslips/group. With respect to tumors, a random sample of 10 fields/section from two sections/tumor and three tumors/group, were assessed. Controls were incubated with secondary antibodies only.

Protein isolation and Western blotting

Expression of OGFr was evaluated in cells and tumors by Western blotting following published procedures.[12] Cells were sheared with a 25 G needle, while tumor tissue was homogenized in RIPA buffer containing a cocktail of protease and phosphatase inhibitors (Roche, Indianapolis, IN, USA). Equal amounts of protein (60 μg) were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with an antibody to OGFr (1:200). Optical densities (QuickOne; Sigma-Aldrich) were normalized to β-actin (1:5000; Sigma-Aldrich) from the same blot. The percent change in expression was calculated by dividing the normalized values to those of WT samples. Means and SE were determined from two independent experiments.

OGFr-binding assays

Log phase cells in culture were assayed for OGFr using custom synthesized [^{3}H]-[Met^5]-enkephalin (PerkinElmer, Waltham, MA, USA; 52.7 Ci/mmol) following established procedures.[12] Saturation binding isotherms were generated using GraphPad Prism software (GraphPad Prism, La Jolla, CA, USA), and at least three independent assays per clonal line were performed.

Mechanism of modulated growth: DNA synthesis, apoptosis and necrosis

The effect of under-expressing OGFr on DNA synthesis (bromodeoxyuridine [BrdU] incorporation), apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, TUNEL) and necrosis (trypan blue) was evaluated in cells. Cells (5×10^4 cells/coverglass) were grown in culture for 72 h; three hours prior to fixing cells with formalin, 30 μmol/L BrdU was added to these wells.[12] Preparations were either stained with an antibody to BrdU (1:200; Invitrogen)[12,30,31] or processed for TUNEL according to the manufacturer's instruction (Trevigen, Gaithersburg, MD, USA). Necrosis was ascertained in all growth experiments. In cells, the proportion of those positive for BrdU, TUNEL or trypan blue staining was determined for at least 500 cells/coverslip on two coverslips/group.

Statistical analysis

Tumor incidence was analyzed by the chi-squared test. All other data were analyzed using one-way analysis of variance, with subsequent comparisons made with the Newman–Keuls tests (GraphPad Prism). In some cases, data were evaluated by unpaired t-tests. P values <0.05 were considered significant.

Results

Establishment and characterization of the OGFr shRNA clones

Two SKOV-3 clones, SH-6 and SH-16, stably transfected with OGFr shRNA were expanded and characterized by semi-quantitative immunohistochemistry, Western blotting, OGFr binding and growth (Figures 1 and 2). In all studies, comparisons were made to (and between) WT and EV cells.

OGFr protein is under-expressed in transfected cell lines

Semi-quantitative immunohistochemistry of OGFr revealed a marked decrease in OGFr immunofluorescence (mean gray value) in clonal cell lines SH-6 (23%) and SH-16 (27%) relative to WT cells (Figures 1a and b). For all cultures, OGFr was visible in the cytoplasm, and a speckling of immunoreactivity was often noted in cell nuclei. Cells processed with only secondary antibody showed no staining (Figure 1a, inset). Western blot analysis of SH-6 and SH-16 clonal cell lines indicated that the 62-kDa band of OGFr, standardized for loading with actin, was decreased 73% and 56%, respectively, relative to WT cells (Figure 1c). No differences in OGFr expression were observed between WT and EV groups.

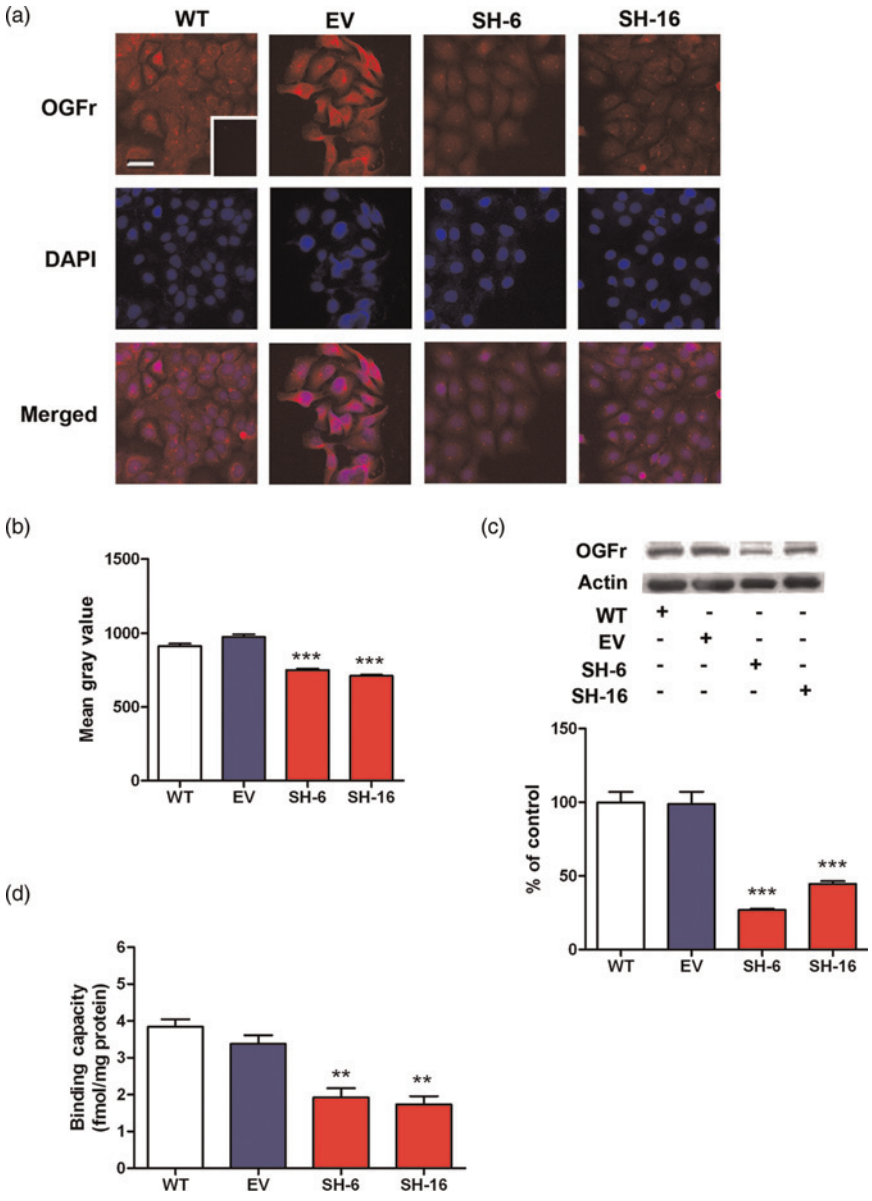


Figure 1 The expression and distribution of OGFr in SKOV-3 cells transfected with OGFr shRNA. (a) Photomicrographs of untransfected wild-type cells (WT), cells transfected with an empty vector (EV), or clonal cell lines SH-6 and SH-16 stained with antibodies (1:200) to OGFr; photomicrographs were taken at the same exposure time. Rhodamine-conjugated IgG (1:1000) served as the secondary antibody (red) and nuclei were visualized with DAPI (blue). Preparations incubated with secondary antibodies only (inset). Bar=10 μm. (b) Semi-quantitative measurement of OGFr-staining intensity (mean gray value) from at least 100 cells/group utilizing three coverslips/group. (c) Western blot of OGFr and densitometric analysis normalized to β-actin from two independent experiments. (d) Saturation isotherms calculating binding capacity (B_{max}) of radiolabeled OGF for at least three independent assays performed in duplicate. Data in (b), (c) and (d) represent means $\pm$ SE. Significantly different from WT at $^{**}P < 0.01$ and $^{***}P < 0.001$. OGFr, opioid growth factor receptor; DAPI, 4′,6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal)

Under-expression of OGFr downregulates receptor binding capacity

To further characterize the OGFr under-expressing clones, OGFr-binding capacity (B_{max}) and binding affinity (K_d) were determined. Binding assays of radiolabeled [Met^5]-enkephalin revealed specific and saturable binding in the nuclear fraction of all cells. Binding capacity (fmol/mg protein) for clonal cells was markedly reduced compared with those of WT (3.8 ± 0.2) and EV (3.4 ± 0.2) groups, with binding capacities of 1.9 ± 0.2 and 1.7 ± 0.2 recorded in SH-6 and SH-16 clones, respectively (Figure 1d). Binding affinity did not differ between groups (data not shown).

Under-expression of OGFr upregulates cancer cell proliferation

The repercussion of under-expressing OGFr was determined by evaluating cell growth over 96 h. Cell number was increased in SH-6 (48–132%) and SH-16 (33–88%) cultures, compared with EV and WT controls (Figure 2a). Calculation of doubling times based on multiple growth curves revealed decreases in clonal cell lines SH-6 (35%) and SH-16 (29%) relative to WT and EV controls (Figure 2b). No differences in doubling times were noted between cells in WT and EV cultures (~39 h).

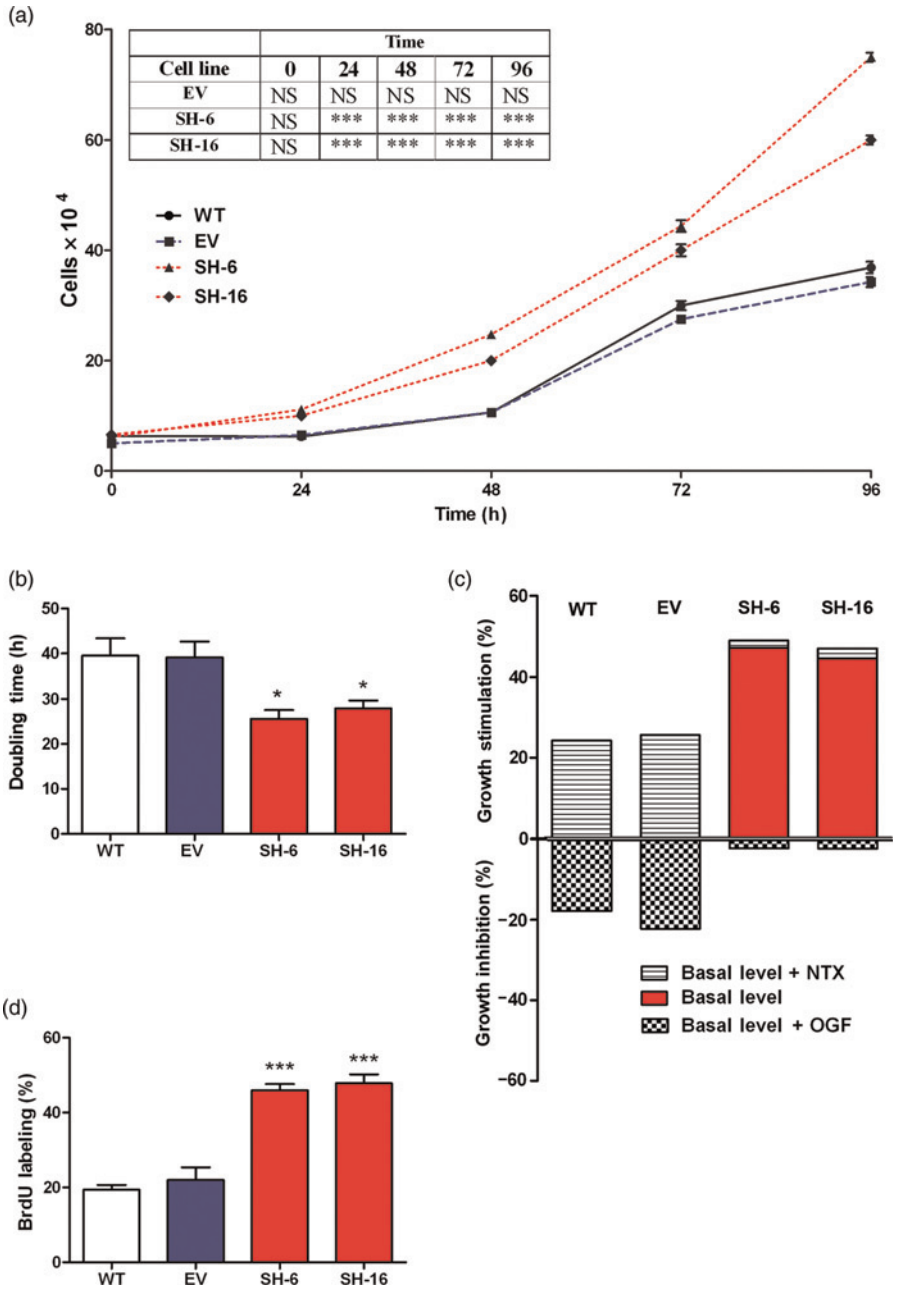


Figure 2 Clones under-expressing OGFr are increased in cell proliferation and cannot be modulated by OGF or NTX. (a) Growth curves of SH-6, SH-16, WT and EV cells from at least 2 aliquots/well and 2 wells/time point. Inset provides levels of significance. (b) Doubling times calculated from at least 2 growth curves/cell line and analyzed by linear regression. (c) The growth effects of exogenous OGF (10^{-6} mol/L), NTX (10^{-6} mol/L) or an equivalent volume of sterile water (basal level) on WT, EV, SH-6 and SH-16 cell lines. Compounds were replaced daily. (d) Quantification of DNA synthesis (% BrdU incorporation) in cells for at least 10 fields/coverslip from two coverslips/cell line. Medium was changed daily in all experiments. Values for (a), (b) and (d) represent means $\pm$ SE; significantly different from WT at $^{*}P < 0.05$ and $^{***}P < 0.001$. Values in (c) are expressed as % growth inhibition or stimulation, compared with WT basal levels. OGF, opioid growth factor; OGFr, opioid growth factor receptor; NTX, naltrexone; NS, not significant. (A color version of this figure is available in the online journal)

Exogenous OGF or NTX have no effect on cell proliferation in cultures under-expressing OGFr

The ability of exogenous OGF (10^{-6} mol/L) or NTX (10^{-6} mol/L) to modulate cell number in clonal cell lines under-expressing OGFr was evaluated. Following 96 h of treatment, cell number was reduced in WT (18%) and EV (22%) cultures administered OGF, and increased in WT (25%) and EV (26%) cells treated with NTX, compared with WT and EV cells subjected to sterile water (Figure 2c). In contrast, clonal cell lines SH-6 and SH-16 had a comparable number of cells regardless of treatment with OGF, NTX or sterile water (Figure 2c).

OGFr under-expression increases DNA synthesis without altering cell survival

To evaluate the mechanism by which OGFr under-expression increased cell number, DNA synthesis and cell survival were evaluated. Compared with the labeling indexes of WT (19%) and EV (22%) cultures, clones SH-6

and SH-16 had 136% and 146%, respectively, more cells incorporating BrdU (Figure 2d). Examination of apoptosis (TUNEL) or necrosis (trypan blue staining) in SH-6 and SH-16 clonal cell lines revealed less than 0.1% positive cells for apoptosis or necrosis; these data were comparable with that of WT and EV groups (data not shown).

OGF is present but expression levels unchanged in clonal cell lines under-expressing OGFr

OGF was visible in the cytoplasm, with a speckling of immunoreactivity observed in cell nuclei (Figure 3a). No staining was detected in cells processed with secondary antibody only (Figure 3a, inset). OGF immunofluorescence (mean gray value) did not differ between groups (Figure 3b).

Preliminary tumor burden study: under-expression of OGFr in human ovarian cancer xenografts accelerates tumor appearance

An initial evaluation of latency to the development of measurable tumors (i.e. $\geq$62.5 mm^3) revealed that mice receiving 1×10^6 or 2×10^6 EV cells had latencies of 26.7 ± 3.6 and 23.5 ± 2.5 d, respectively (Figures 4a and b). Mice inoculated with 1×10^6 SH-6 or SH-16 cells had latencies that were 21 and 14 d shorter (i.e. days 5 and 12), respectively, than mice receiving 1×10^6 EV cells. Animals administered 2×10^6 SH-6 or SH-16 cells had latencies that were 22 and 7 d shorter than mice inoculated with 2×10^6 EV cells (Figures 4a and b). Comparable latencies were noted in mice injected with 1×10^6 or 2×10^6 WT or EV cells (Figures 4a and b). Latencies to tumor development were similar in animals inoculated with 4×10^6 WT, EV, SH-6 or SH-16 cells (data not shown).

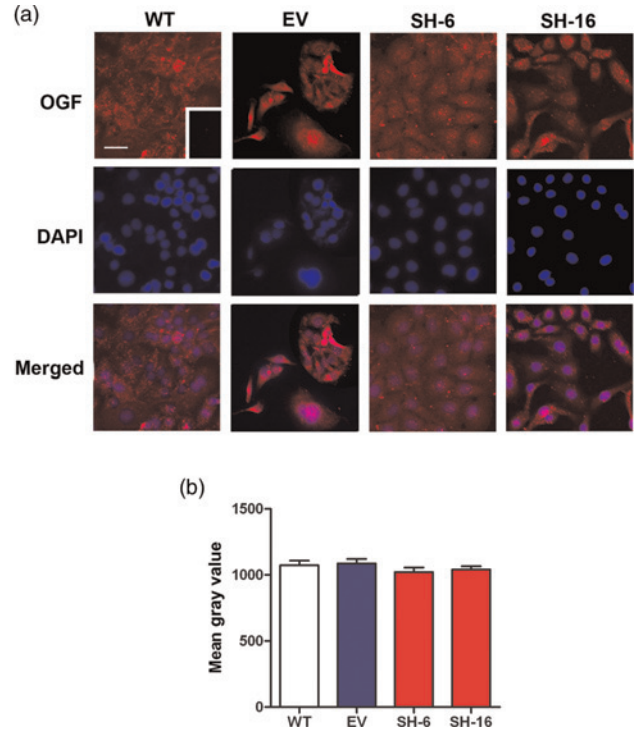


Figure 3 The expression and distribution of OGF in SKOV-3 cells transfected with OGFr shRNA. (a) Photomicrographs of untransfected wild-type cells (WT), cells transfected with an empty vector (EV) or SH-6 and SH-16 cell lines stained with an antibody to OGF (1:200). All photomicrographs were taken at the same exposure time. Rhodamine-conjugated IgG (1:1000, red) served as the secondary antibody, and cell nuclei were visualized with DAPI (blue). Preparations incubated with secondary antibodies only (inset). Bar=10 μm. (b) Semi-quantitative measurement of OGF staining intensity (mean gray value) from at least 100 cells/group and three coverslips/group. Data represent means $\pm$ SE. OGF, opioid growth factor; OGFr, opioid growth factor receptor; DAPI, 4′,6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal)

Mice with xenografts under-expressing OGFr have increased tumor incidence, accelerated tumor appearance, increased tumor volumes and fail to respond to OGF

In order to (i) confirm the findings in the preliminary tumor burden study; (ii) determine the effects of molecular under-expression of OGFr on tumor incidence and tumor volume; and (iii) assess whether OGF could modulate tumors under-expressing OGFr, mice inoculated with 1×10^6 WT, EV or SH-6 cells were injected on a daily basis with OGF (10 mg/kg) or an equivalent volume of saline when tumors became visible. In contrast to WT and EV groups receiving saline, which contained no mice with measurable tumors on day 4, 58% of mice in the SH-6 group and receiving saline had measurable tumors (Figure 5a). On day 5, when 83% of the mice in the SH-6 group had measurable tumors, only 12% and 8% of mice in the WT and EV groups, respectively, had tumors. By day 7, when 100% of mice injected with SH-6 cells and saline had measurable tumors, only 25% of WT and 16% of EV mice had measurable tumors. On days 19 and 21, 100% of the mice in the WT and EV groups, respectively, had measurable tumors, in contrast to the SH-6 group that reached a 100% incidence on day 7. The latency to appearance of a measurable tumor for mice injected with WT (11.9 ± 1.5 d) or EV (13.0 ± 1.5 d) cells and exposed to saline was comparable, but was significantly decreased by nine days in mice injected with SH-6 cells and treated with saline (Figure 5b). Evaluation of tumor volumes in mice injected with SH-6 cells and subjected to saline revealed that tumor volumes were elevated 41–267% on days 4–14 relative to mice inoculated with WT cells and receiving saline (Figure 5c). Even within one day after tumor cell inoculation, all animals injected with SH-6 cells had visible tumors (i.e. diameter of 3 mm), whereas none of the animals in either the WT or EV groups had visible tumors on day 4. The addition of injections of OGF to mice inoculated with SH-6 cells made no difference in tumor kinetics as compared with cohorts receiving SH-6 cells and saline, but exposure of WT or EV groups to OGF revealed a marked depression in tumor growth beginning on day 14 in comparison with animals in the WT or EV groups receiving saline. Figure 5c also indicates that tumor growth in the mice receiving SH-6 cells (with or without OGF) reaches its optimal size by day 4 after tumor cell injection, and remains in a plateau thereafter for the next two and one-half weeks.

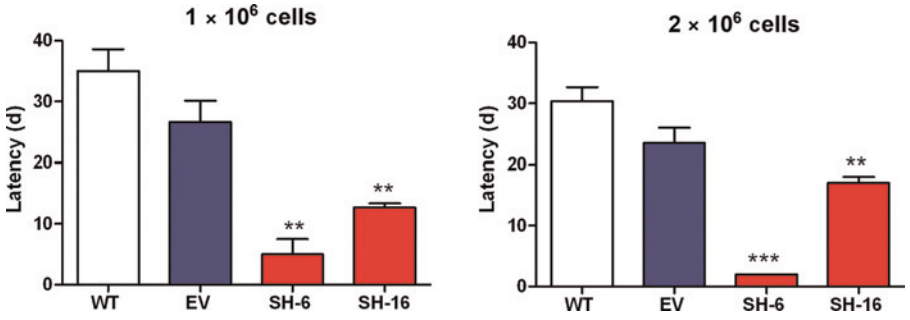


Figure 4 Preliminary tumor burden study: OGFr under-expression decreases the latency to the development of measurable tumors. (a, b) Latency (days) to the development of a measurable ($\geq$62.5 mm^3) tumor in mice receiving subcutaneous inoculation of 1 $\times$ 10^6 (a) or 2 $\times$ 10^6 (b) WT, EV, SH-6 or SH-16 cells. Data represent means $\pm$ SE for three animals/group. Significantly different from mice receiving WT cells at $^{**}P < 0.01$ and $^{***}P < 0.001$. OGFr, opioid growth factor receptor; WT, wild-type; EV, empty vector. (A color version of this figure is available in the online journal)

On the other hand, mice in the WT and EV groups exhibit a slow increase in tumor size during this period.

Compared with saline-administered WT or EV controls, mice inoculated with WT or EV cells and treated with OGF had reduced tumor volumes (38–60%) beginning on day 14 (Figure 5c), and decreased terminal tumor weights (32–55%) (Figure 5d). Tumor volumes and terminal tumor weights were comparable in mice inoculated with SH-6 cells that were treated with OGF relative to mice injected with SH-6 cells receiving saline (Figures 5c and d). Terminal spleen and body weights were comparable regardless of the cell line inoculated, as well as with respect to OGF or saline treatment (data not shown).

OGFr under-expression is maintained in xenografts

To examine OGFr distribution and expression in the transition from *in vitro* to *in vivo* environments, semi-quantitative immunohistochemistry and Western blot were performed on tumors taken from mice inoculated with WT, EV or SH-6 cells. The location of OGFr was similar in all groups, with immunoreactivity detected in the cytoplasm and a speckling noted in cell nuclei (Figure 6a). Tumors processed only with secondary antibody showed no staining (Figure 6a, inset). Compared with the WT group, OGFr expression in SH-6 xenografts was decreased as noted by semi-quantitative immunohistochemistry (35%) (Figure 6b) and Western blot (67%) (Figures 6c and d).

OGF expression is unchanged in xenografts under-expressing OGFr

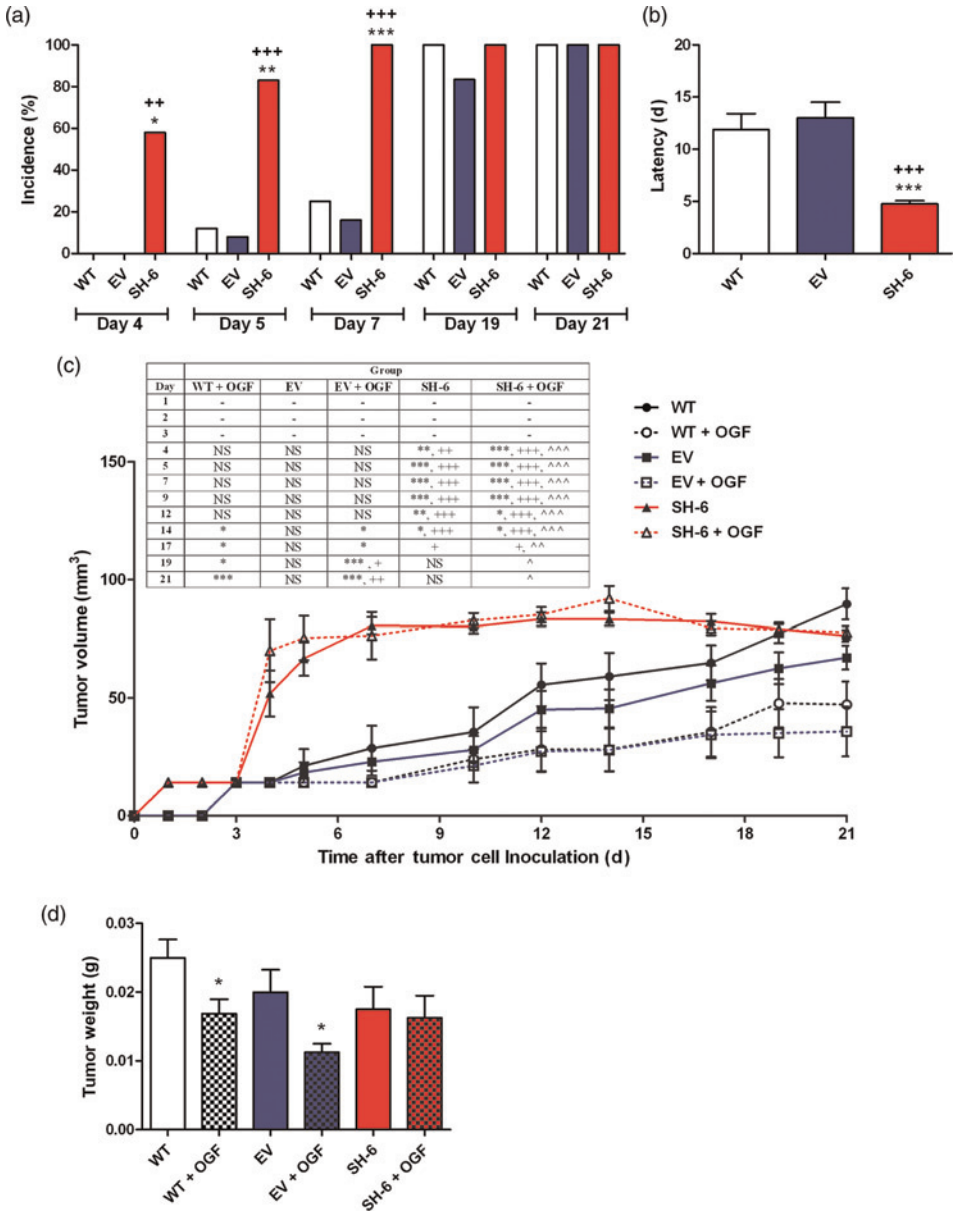
To ascertain OGF distribution and expression in xenografts with decreased OGFr, semi-quantitative immunohistochemistry was performed using an antibody to OGF. OGF was visible in the cytoplasm, with some immunoreactivity often noted in cell nuclei (Figure 6e). Tumors processed with only secondary antibody showed no staining (Figure 6e, inset). OGF distribution and immunofluorescence (mean gray value) did not differ between groups (Figures 6e and f).

Discussion

This study is the first to report the stable under-expression of OGFr in a cancer cell line, and reveals that diminishing OGFr in human ovarian cancer cells markedly affects cell proliferation and tumorigenesis. *In vitro*, attenuation of OGFr resulted in increases in cell number and DNA synthesis, along with decreases in doubling times. Cultures with a deficit of OGFr did not respond to challenges with an opioid agonist (OGF) or antagonist (NTX), in contrast to WT or EV cultures that were inhibited or increased by exposure to OGF and NTX, respectively. These findings in tissue culture were extended into animals using subcutaneous tumor transplantation, and revealed that OGFr under-expression increases tumor incidence, decreases the latency to development of measurable tumors and increases tumor volumes. Thus, under both *in vitro* and *in vivo* conditions, the effects of a loss of OGFr were maintained, indicating that there was no tolerance or compensation to the repercussions of decreasing OGFr in these cells.

Knockdown of OGFr was confirmed in cells and tumor tissue by a combination of receptor-binding assays, semi-quantitative immunohistochemistry and Western blot analysis. Binding capacity of OGFr was markedly reduced in clonal cell lines under-expressing OGFr in comparison with WT and EV cells. However, no changes in binding affinity were noted, demonstrating that there was no compensatory action (i.e. increase in binding affinity) of the decreased OGFr. Furthermore, the vector did not contribute to OGFr knockdown, as WT and EV cells were similar in OGFr expression and binding, as well as in growth (*in vitro* and *in vivo*). Additionally, the characterization of two clones ensured that the outcomes of transfection were not related to an aberrancy of only a singular clone, but rather were representative of the repercussions from under-expression of OGFr.

Although the methodology utilized in these studies diminished OGFr by approximately 70%, this extent of under-expression did have a significant effect on function. For example, cells with a reduction in OGFr were increased in BrdU-labeling index by more than 1.4-fold from WT cells. Moreover, tumor incidence was notably increased in mice inoculated with cells under-expressing OGFr, with 100% of these mice having measurable tumors within one week



Day	WT + OGF	EV	EV + OGF	SH-6	SH-6 + OGF
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	NS	NS	NS	**, ++	***, +++, ^^^
5	NS	NS	NS	***, +++	***, +++, ^^^
7	NS	NS	NS	***, +++	***, +++, ^^^
9	NS	NS	NS	***, +++	***, +++, ^^^
12	NS	NS	NS	**, +++	*, +++, ^^^
14	*	NS	*	*, +++	*, +++, ^^^
17	*	NS	*	+	+, ^^
19	*	NS	***, +	NS	^
21	***	NS	***, ++	NS	^

Figure 5 Repercussion of under-expressing OGFr on tumor progression and response to OGF treatment. Mice were inoculated subcutaneously with 1×10^6 WT, EV or SH-6 cells and treated daily with OGF (10 mg/kg) or an equivalent volume of saline when tumors became visible. (a) Incidence of measurable ($\geq$62.5 mm^3) tumors at 4, 5, 7, 19 and 21 d following tumor cell inoculation in saline-treated mice. (b) Latency (days) to the development of measurable tumors in mice administered saline. (c) Tumor volume in mice that were treated with OGF or an equivalent volume of saline when tumors were visible. Tumors that were visible, but not yet measurable, were estimated to be 3 mm in length and width. (d) Terminal tumor weight. Data in (a–d) represent means $\pm$ SE for 8–12 animals/group. Significantly different from WT at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, from EV at $+P < 0.05$, $++P < 0.01$ and $+++P < 0.001$, and from the WT group treated with OGF at $^{\wedge}P < 0.05$, $^{\wedge\wedge}P < 0.01$ and $^{\wedge\wedge\wedge}P < 0.001$. OGF, opioid growth factor; OGFr, opioid growth factor receptor; WT, wild-type; EV, empty vector; NS, not significant. (A color version of this figure is available in the online journal)

compared with almost three weeks for the WT and EV groups. Therefore, the magnitude of under-expression achieved in this study was sufficient to evoke a physiological effect *in vitro* and *in vivo*. Whether a complete knockout of OGFr in ovarian cancer cells would even further hasten cell proliferative events and tumorigenesis in comparison with controls remains to be investigated.

Because of the marked acceleration in proliferation of cells under-expressing OGFr, our strategy for tumor studies was to use a low tumor burden in order to provide a window to assess changes for comparison to the WT and EV groups. A lower tumor burden with SH-6 cells resulted in a plateau effect of tumor size. This was evident in the time to reach maximal tumor volume, with mice inoculated with cells under-expressing OGFr reaching this maximal measure on day 4, in contrast to WT and EV groups that took five times longer to attain a comparable tumor size. However, since tumors in the SH-6 group did not increase in size beyond day 4, in contrast to the slow increase in tumor size of the WT and EV mice, a low tumor burden does not explain the difference in tumor kinetics. Thus, diminishing OGFr by molecular engineering in these human ovarian cancer cells must alter other processes involved in tumorigenicity that need to be clarified.

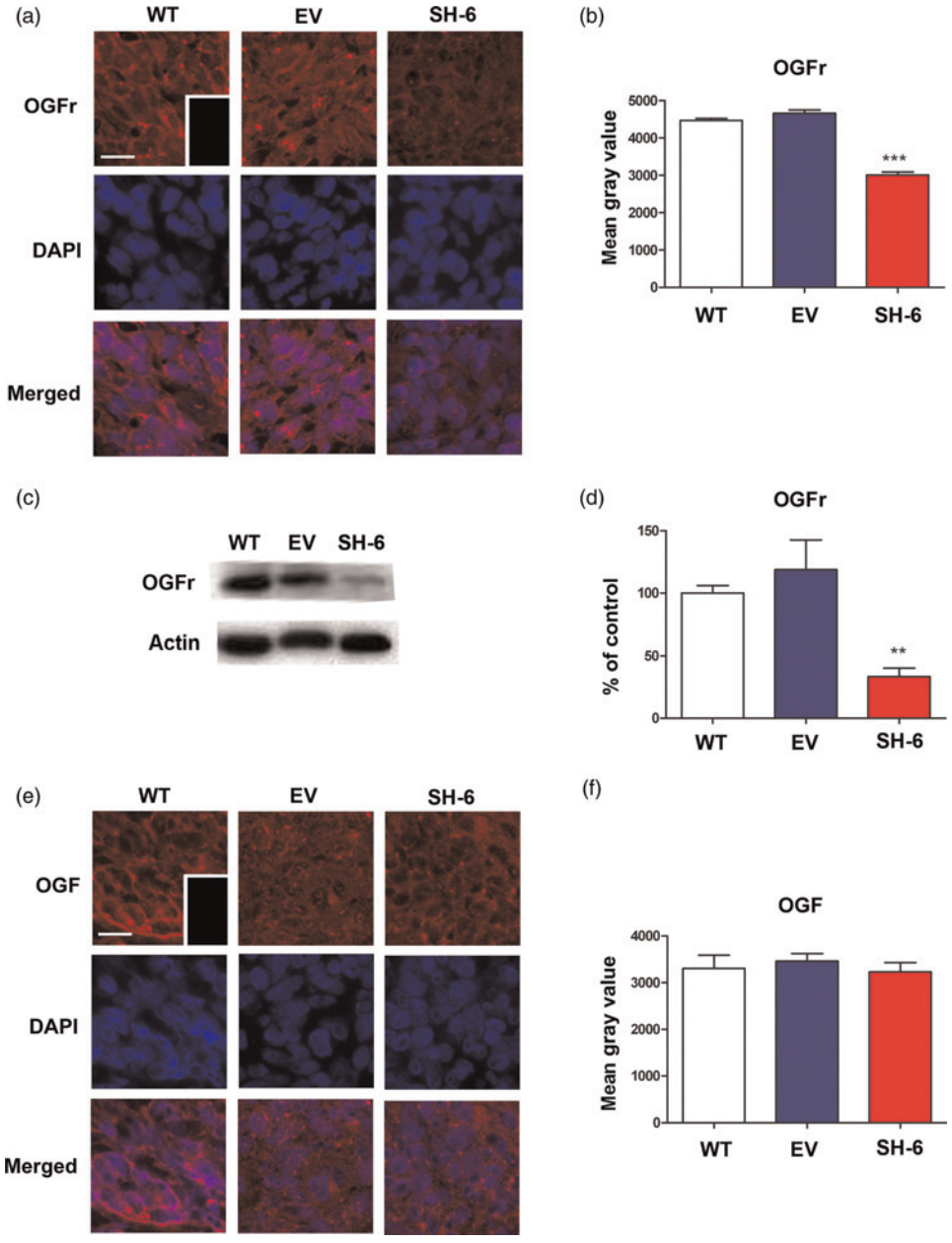


Figure 6 Distribution and expression of OGFr and OGF in subcutaneous tumors. Tumors were assessed 21 d after mice were inoculated with WT, EV or SH-6 cells. (a, e) Photomicrographs of tumors stained with antibodies (1:200) to OGFr (a) or OGF (e). All photomicrographs were taken at the same exposure time. Rhodamine-conjugated IgG (1:1000) served as the secondary antibody, and cell nuclei visualized with DAPI. Preparations incubated with secondary antibodies only (a, e insets). Bar=10 μm. (b, f) Semi-quantitative measurement of OGFr (b) and OGF (f) staining intensity (mean gray value) from 10 fields/section, two sections/tumor and three mice/group. (c) Western blot of OGFr expression in mice inoculated with SH-6, WT and EV cells. (d) Densitometric analysis of Western blots normalized to β-actin from 2 independent experiments. Data represent means $\pm$ SE. Significantly different from WT at $^{**}P < 0.01$ and $^{***}P < 0.001$. OGF, opioid growth factor; OGFr, opioid growth factor receptor; DAPI, 4′,6-diamidino-2-phenylindole; WT, wild-type; EV, empty vector; IgG, immunoglobulin G. (A color version of this figure is available in the online journal)

Because OGF has been identified as the opioid peptide that binds to OGFr to repress ovarian cancer cell proliferation,[12] one can ask whether the growth acceleration recorded with a reduction in OGFr could be the result, at least in part, to a compensatory decrease in OGF. The data gathered in this study do not support this hypothesis. First, OGF expression as measured by semi-quantitative immunohistochemistry was similar in cultures and tumors under-expressing OGFr to those of WT and EV cells/tissues. Second, even when exogenous OGF was added to cells in culture with diminished OGFr, as well as animals with xenografts of these cells, cell number and tumor volumes remained elevated compared with cells/animals in the WT and EV groups. Moreover, mice with under-expression of OGFr and receiving OGF were comparable in tumor progression to mice with a diminishment of OGFr and treated with vehicle. This was distinct from the marked decreases in cell number and tumor volumes in WT and EV groups exposed to OGF wherein reductions of up to 60% were recorded. These data demonstrate that there is a loop between OGF and OGFr that is critical to regulating cell number in ovarian cancer.

To examine the mechanism by which OGFr underexpression accelerates cell number in tissue culture, cell survival and DNA synthesis were monitored. No changes in the number of apoptotic or necrotic ovarian cancer cells with a molecular attenuation of OGFr were discerned in contrast to WT and EV cells. Thus, alteration in cell survival pathways did not account for the acceleration in cell number. However, DNA synthesis was markedly elevated in cells with a deficiency in OGFr compared with WT or EV controls. These data on ovarian cancer are consistent with previous reports on other cancers documenting a mechanism targeted to cell proliferation.[32–35]

The present findings reveal an acceleration of cell proliferation and tumorigenesis with knockdown of OGFr, demonstrating the tonic activity of the OGF–OGFr axis in human ovarian cancer. A continuously active loop between native OGF and OGFr has been recorded earlier in tissue culture of ovarian cancer with (i) continuous blockade of OGF–OGFr with NTX; (ii) neutralization of native OGF with an antibody to OGF; and (iii) transient knockdown of OGFr with siRNA, all markedly increasing cell proliferation.[12,23] Additional evidence gathered in these experiments found that diminishment of OGFr in ovarian cancer disengaged and attenuated peptide–receptor interfacing under *in vitro* conditions, thereby rendering cells insensitive to the growth modulatory effects of exogenous OGF (i.e. inhibition) and NTX (i.e. stimulation). Moreover, tumors generated from cells under-expressing OGFr were not responsive to the effects of OGF administration. Thus, molecular engineering of an underexpression of OGFr has extremely specific and focused repercussions on the replication of ovarian cancer cells, as well as on tumorigenic events.

The present study clearly reveals that the OGF–OGFr axis is a fundamental biological pathway that impacts on the course and progression of human ovarian cancer. This raises the question as to the repercussions of potential alterations (e.g. mutation, processing) of OGF and/or OGFr on the growth of ovarian tumors. Moreover, since the OGF–OGFr system involves other biological processes (e.g. nucleocytoplasmic transport), dysfunction of one or more of these pathways could impair regulation of cell proliferation – and tumorigenesis – in patients with ovarian cancer. These findings that the OGF–OGFr axis is of vital importance to advancement of ovarian cancer, implies that strategies to harness this peptide–receptor system offers a distinct means to restore homeostatic conditions. Modulation of the OGF–OGFr axis can be achieved by a variety of means, including (i) administration of exogenous OGF;[32–35] (ii) the use of imiquimod to upregulate OGFr[36]; and (iii) treatment with low-dose NTX (LDN) which upregulates both OGF and OGFr and, after drug is no longer present, allows for a robust functional effect by reducing cell proliferation.[37–40] Indeed, both OGF[12,23–25] and LDN[24,41,42] have been reported to markedly suppress the course of human ovarian cancer. Of course, as documented herein for ovarian cancer, as well as in previous reports[12,23–25,41,42], all these strategies are dependent on a functioning OGF–OGFr system.

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