

# Neuregulin Expression, Function, and Signaling in Human Ovarian Cancer Cells

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## ABSTRACT

**Purpose:** To investigate the expression and function of neuregulin (NRG) isoforms in ovarian cancer cell lines and tumor samples.

**Experimental Design:** Expression of NRG-1 $\alpha$  and NRG-1 $\beta$  proteins were detected by immunohistochemistry and mRNA by RT-PCR. erbB receptor levels and downstream signaling proteins were measured by Western blot analysis.

**Results:** Expression of NRG-1 $\alpha$  and NRG-1 $\beta$  proteins were detected by immunohistochemistry in 46 of 53 (87%) and 41 of 53 (77%) ovarian carcinomas, respectively. Serous carcinomas express higher levels of NRG-1 $\alpha$  than endometrioid carcinomas ( $P = 0.017$ ). NRG mRNA was detected by RT-PCR in 20 of 24 (83%) of ovarian carcinomas and eight of nine (89%) ovarian cancer cell lines. NRG-1 $\alpha$  stimulated the growth of 5 of 14 cell lines whereas NRG-1 $\beta$  stimulated 7 of 14 cell lines. The magnitude of NRG growth response was significantly associated with erbB2 expression levels. NRG-1 $\alpha$  and -1 $\beta$  (1 nM) growth-stimulated cell lines PE01 and PE06 demonstrated increased tyrosine phosphorylation of erbB2 and elevated tyrosine phosphorylation of ERK1 and ERK2. In contrast, the SKOV-3 cell line, the growth of which was unaffected, did not show these downstream responses. An anti-erbB3 receptor antibody (clone H3.105.5) blocked NRG-1 $\beta$  growth changes and signaling in these cell lines. Conversely, the anti-erbB4 antibody (clone H4.72.8) enhanced NRG- $\beta$ 1 growth stimulation. Herceptin also inhibited growth.

**Conclusions:** With NRG expression in the majority of ovarian carcinomas and cell lines, there is the potential for autocrine regulation of cell growth. Interfering with ligand-receptor interactions by receptor blocking antibodies suggests erbB3 is primarily involved in NRG-1 $\beta$ -induced proliferation, with erbB4 having a more complex role.

## INTRODUCTION

NRG<sup>4</sup>, or heregulin, is a member of a complex ligand-binding family originally identified in a search for activators of erbB2 (p185/HER-2/neu; Ref. 1). Independent efforts to identify erbB2 activators identified a series of related splice variants that were named NDF (neu differentiation factor; Ref. 2), ARIA (acetylcholine receptor-inducing activity; Ref. 3), GGF (glial growth factor; Ref. 4), and SMDF (sensory and motor neuron-derived factor; Ref. 5) and collectively as the NRGs (4). As these various names suggest, the NRGs are implicated in a variety of cell processes including cell proliferation, growth inhibition, differentiation, chemotaxis, and migration. Variations in the EGF-like domain of the NRG structure give rise to  $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms whereas differences in the juxtamembrane segment generate five subtypes designated 1 to 5.

NRG-induced cell responses are mediated by the erbB family of tyrosine kinase receptors. Although NRG was shown to activate erbB2, it does not induce erbB2 dimerization and was subsequently shown to interact with homodimers of either erbB3 or erbB4 or with heterodimers of erbB2 with erbB3 or erbB4 (6–8). Intracellular signaling, then, involves a variety of pathways including the Ras/Erk and PI3-kinase cascades (9).

The roles for EGF receptor and erbB2 as growth mediators in ovarian cancer are well established, however, the functions of erbB3 (HER-3) and erbB4 (HER-4) are less clearly defined. Overexpression of the EGF receptor and erbB2 in ovarian carcinomas has been associated with poor survival (10–13), and many reports have demonstrated growth stimulation by EGF or TGF- $\alpha$  in ovarian cancer cell lines (14–16). We have previously demonstrated expression of erbB3 (17–19) and erbB4 in the majority of ovarian cancers and observed that erbB4 is expressed as multiple splice variants in this disease (20).

In this study, we describe the expression, function, and signaling associated with two major forms of NRG, namely NRG-1 $\alpha$  and NRG-1 $\beta$ , in a large panel of ovarian cancer cell lines and primary tumors. No data were available on the expression of the NRG isoforms in primary ovarian cancers, and we have determined the expression patterns in different histological subtypes. We have then related this expression to that of the presence of its receptors. NRG functionality was then explored

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<sup>4</sup> The abbreviations used are: NRG, neuregulin; ERK, extracellular related kinase; EGF, epidermal growth factor; TGF, transforming growth factor.

in cell line models, and although several studies using a limited number of ovarian cancer cell lines have previously explored the effects of NRG on ovarian cancer cell growth, these studies have produced conflicting results that may be dependent on the form of NRG studied and assay conditions (21–25). We then sought to associate the magnitude of ligand-induced growth effects to the level of erbB expression and monitored intracellular signaling. Finally, using antibodies that block the interaction of NRG with erbB3 or erbB4 receptor, we assessed the specific involvement of each receptor in signaling and function.

## MATERIALS AND METHODS

**Cell Lines.** The PE01, PE01<sup>CDDP</sup>, PE04, PE06, and PE014 cell lines were developed at the Edinburgh Medical Oncology Unit (26). The OVCAR-3, OVCAR-4, and OVCAR-5 cell lines were kindly donated by Dr. T. Hamilton (Fox Chase Institute, Philadelphia, PA). The 41M, OAW-42, and 59M cell lines were obtained from the European Tissue collection (Porton Down, United Kingdom). The SKOV-3, CAOV-3, and SW626 cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were routinely cultured at 37°C, 90% humidity, and 5% CO<sub>2</sub> in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) containing 10% heat-inactivated FCS, streptomycin (100 µg/ml), and penicillin (100 IU/ml).

**Tumor Samples.** Fresh primary ovarian tumor tissue was obtained from 53 patients with epithelial ovarian cancer at initial debulking surgery, transferred to liquid nitrogen, then formalin fixed and embedded in paraffin. Tumor histology was assessed on paraffin-embedded sections and classified according to WHO criteria. Tumor histologies were classified as: serous adenocarcinoma (29 tumors), endometrioid adenocarcinoma (18 tumors), and clear cell carcinomas (6 tumors). Information on stage was available for 48 patients and on grade of differentiation for 44 patients.

**Immunohistochemistry.** Sections (3 µm) were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubating sections in 3% H<sub>2</sub>O<sub>2</sub> for 30 min. Sections were immersed in citric acid buffer (0.005 M, pH 6.0) and microwaved for 3 × 5 min. Slides were washed in 0.05 M Tris/NaCl buffer (pH 7.6) and then incubated in 20% FCS for 10 min. Primary antibodies were added for 1.5–2 h. The rabbit polyclonal antibodies HRG 76 and HRG 102 (diluted 1:20) were used to detect NRG-1α and NRG-1β forms, respectively (27). After primary antibody incubation, sections were washed in Tris/NaCl buffer. A streptavidin-biotin multilink method (Str-AviGen Multilink kit; Biogenex, San Ramon, CA) was used for detection of reactivity. Sections were stained with secondary multilink antibody (1:20 dilution for 30 min), followed by horseradish-peroxidase-labeled streptavidin complex (1:20 dilution for 30 min). Diaminobenzidine tetrachloride was used as chromagen and applied for 5 min. Sections were counterstained in hematoxylin, dehydrated, and mounted. Negative controls for each tumor section were included in all runs by replacing the primary antibody with Tris buffer. Reactivity was measured using a semiquantitative scale with 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) relative to the positive control section. Slides were assessed by two observers (S.P.L. and A.M.)

independently, and assessment was concordant in 90% readings. Where there were differences in the magnitude, these were reviewed by the two observers to obtain an agreed score.

**RT-PCR.** Total cellular RNA was extracted from cells in log phase growth or from primary ovarian cancers using TRI reagent (Sigma Chemical Co., Poole, United Kingdom). Samples were treated with 20 units/50 µl DNase I (Boehringer Mannheim, East Sussex, United Kingdom) to remove genomic DNA contamination. RNA was then reextracted using a phenol/chloroform protocol. Reverse transcription was performed with a first-strand cDNA Synthesis kit (Boehringer Mannheim) using the oligo dT primer provided. One microgram of RNA yielded 20 µl of cDNA, of which 2.5 µl was used for each subsequent PCR reaction with each primer pair. PCR reactions were performed in a final volume of 25 µl containing the following: 1× PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mixture, 1.25 U Taq polymerase (ICRF Clare Hall, South Mimms, United Kingdom), 400 mM each primer (ICRF Clare Hall). The PCR conditions for actin were: step 1, 94°C for 2 min; step 2 consisted of 35 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s; step 3, 72°C for 5 min. The following primers were used to detect NRG isoforms, GACCTCTACTTCTCGTGACA and TCCAATCTGTTAGCAATGTG, and for γ-actin, ATGGCATCGTCACCAACTGG and ATGACAATGCCAGTGCG. PCR products were visualized after electrophoresis on polyacrylamide gels by staining with ethidium bromide. Samples were scored as positive when a PCR product of the expected molecular size was amplified and identified following electrophoresis. PCR products were sized using a 100-bp ladder (Life Technologies, Inc., Paisley, United Kingdom), and diethyl pyrocarbonate-treated water was used as a control to detect the presence of contamination in reagents.

**Western Blotting.** Cell lines were grown to 80% confluence in 75-cm<sup>2</sup> flasks (Falcon), in the presence of RPMI 1640 containing 10% FCS. The cells were washed twice in cold PBS, lysed, detached from the flasks, and spun at 15,000 rpm at 4°C. Protein content of the resulting supernatant was determined by Bradford assay and stored at –80°C before Western blotting. Following PAGE, proteins were transferred onto an Immobilon membrane (Millipore, Watford, United Kingdom) using a Bio-Rad Semi-Dry blotting apparatus (30 V overnight). Membranes were blocked in Tris-buffered saline (pH 7.4), containing 0.05% Tween, 5% ovalbumin, and 10% normal sheep serum. Antibodies were incubated for 1 h at room temperature. The antibodies were used at the following concentrations/dilutions: EGF receptor (H11 and 11E8, Neomarkers; 1.5 µg/ml), erbB2 (CB11, Novocastra; 1:40), erbB3 (RTJ.2, Neomarkers; 1:200), erbB4 (Ab-2, Neomarkers; 1:400), phosphotyrosine (PY20, Santa Cruz; 1 µg/ml), phospho-ERK (9101S, New England Biolabs; 1:1000). Detection made use of a chemiluminescence Western blotting kit (Boehringer Mannheim). Quantitative values for the erbB receptor proteins were obtained by densitometric analysis using a gel scanner (UVP Life Sciences, Cambridge, United Kingdom) and analyzed by Labworks gel analysis software (UVP Life Sciences, Cambridge, United Kingdom). This provided integrated absorbance values. In experiments using NRG-1β and the anti-erbB3 and anti-erbB4 antibodies, conditions used were as described for the growth assays.

Table 1 NRG-1 $\alpha$  and NRG-1 $\beta$  expression in primary ovarian cancer

	Staining intensity <sup>a</sup>									
	NRG-1 $\alpha$				<i>P</i> <sup>b</sup>	NRG-1 $\beta$				<i>P</i>
	Negative	Borderline	Weak	Strong		Negative	Borderline	Weak	Strong	
All tumors	1	6	17	29		2	10	21	20	
Stage										
I/II	0	2	5	8	1.00	1	4	6	4	0.34
III/IV	1	3	10	19		1	5	12	15	
Differentiation										
Well/moderate	0	2	8	6	0.065	1	4	8	3	0.057
Poor	1	2	6	19		1	4	9	14	
Histology										
Serous	1	2	7	19	0.017	1	4	11	13	0.55 <sup>c</sup>
Endometrioid	0	3	10	5		0	4	8	6	
Clear cell	0	1	0	5		1	2	2	1	

<sup>a</sup> Homogenous staining was observed in epithelial cells, and the staining intensity is shown.

<sup>b</sup> Fisher's exact test.

<sup>c</sup> Fisher's exact test (serous *versus* endometrioid).

**Growth Assays.** Exponentially growing cells were harvested by trypsinization and plated in 24-well plates at a density of  $2.5 \times 10^4$  cells/well in RPMI 1640 containing 10% FCS. After 24 h to allow for attachment, the medium was removed and replaced with RPMI 1640 without phenol red but containing 5% double charcoal-stripped FCS. After an additional 24 h, NRG-1 $\alpha$  (Sigma Chemical Co.), NRG-1 $\beta$  (Neomarkers), or TGF- $\alpha$  (Boehringer Mannheim) were added at a concentration of  $10^{-9}$  M. This was designated day 0. In experiments where the anti-erbB3 H3.105.5 ( $10 \mu\text{g}/\text{ml}^{-1}$ ; Ab-5, Neomarkers) or anti-erbB4 H4.72.8 antibody ( $10 \mu\text{g}/\text{ml}^{-1}$ ; Ab-3, Neomarkers) were included, these were added 30 min before the addition of NRG-1 $\beta$ . In experiments with herceptin (Trastuzumab; Roche, Welwyn Garden City, Hertfordshire, United Kingdom), this was added 48 h before the addition of NRG-1 $\beta$ . Media with or without NRG-1 $\beta$  and/or antibody were changed on day 2. Cells were harvested from wells on day 5 and counted on a Coulter counter.

**Statistics.** Differences between groups were tested using Students' *t* test and Fisher's test as appropriate. Assessment of associations between erbB receptor expression and magnitude of growth response was by use of the Spearman correlation.

## RESULTS

### NRG Expression in Primary Ovarian Tumors and Cell

**Lines.** Expression of NRG-1 $\alpha$  and NRG-1 $\beta$  immunoreactivity were investigated in 53 ovarian carcinomas and detected in 46 of 53 (87%) and 41 of 53 (77%) ovarian carcinomas, respectively (Table 1). A small proportion of tumors was stained in a borderline manner, and these were classified separately and considered together with the negatively staining tumors. Fifty-five percent showed moderate-strong staining for NRG-1 $\alpha$ , 32% showed weak staining, and 11% showed borderline staining. For NRG-1 $\beta$ , 38% demonstrated moderate-strong staining, 39% weak staining, and 19% borderline staining. Both NRG isoforms were expressed in a diffuse cytoplasmic pattern in the neoplastic epithelial cells, and no stromal staining was observed. Examples are shown in Fig. 1A.

Differential expression of NRG-1 $\alpha$  but not NRG-1 $\beta$  was

observed between the two predominant histologies of ovarian adenocarcinoma, with serous adenocarcinomas more likely to express higher levels of NRG-1 $\alpha$  than endometrioid tumors ( $P = 0.017$ ; Table 1). Neither stage nor grade of differentiation could be associated with the expression level of NRG-1 $\alpha$  or NRG-1 $\beta$ , although grade of differentiation was of near borderline significance ( $P = 0.065$  and  $0.057$  for NRG-1 $\alpha$  and NRG-1 $\beta$  respectively; Table 1).

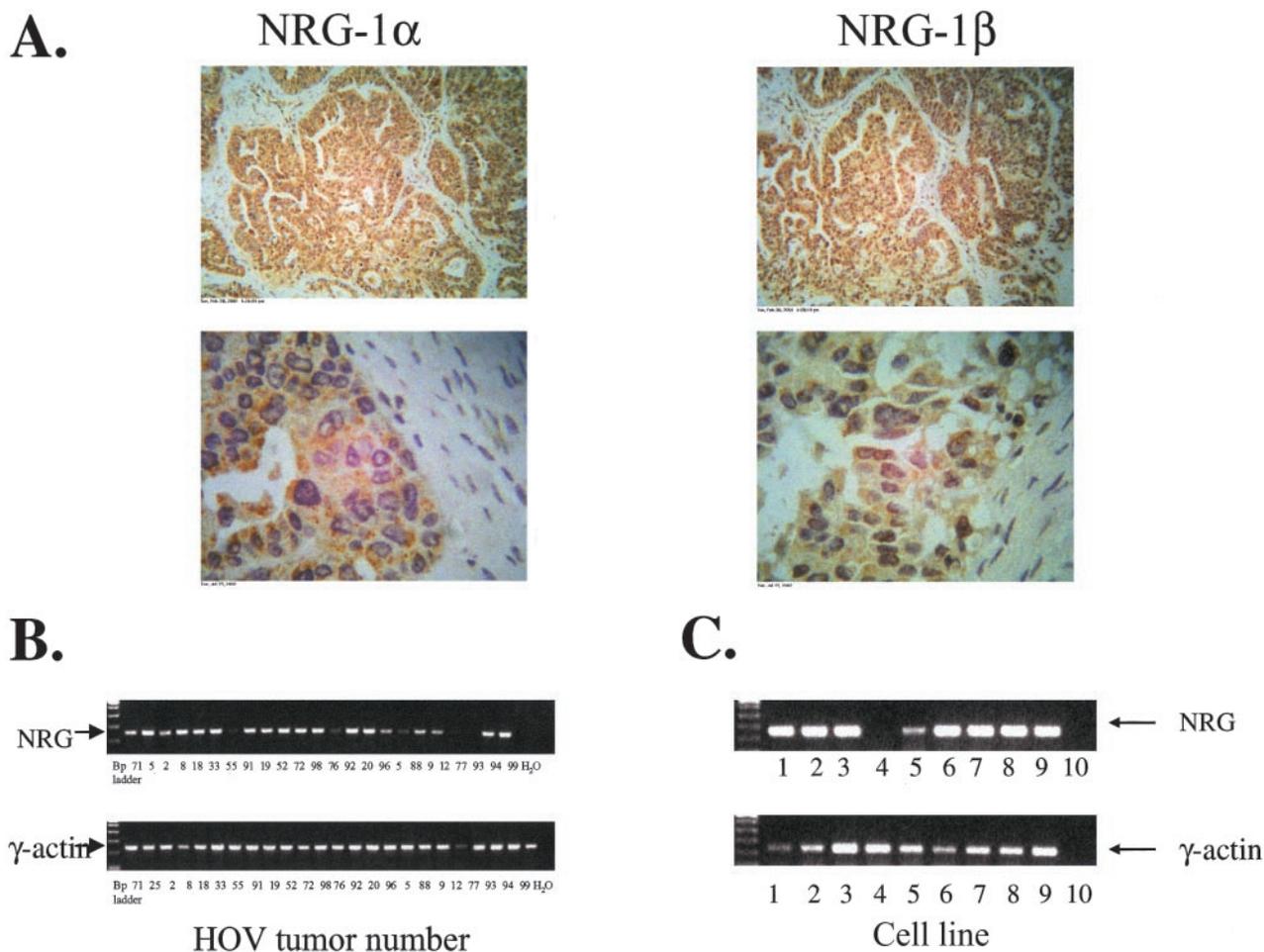
Because the majority of ovarian cancers expressed NRG isoforms, it is feasible that the ligand is acting in an autocrine manner within this disease. The erbB receptor status in this series of cancers had previously been determined (17, 18, 20). Expression of the receptors to which NRG binds (erbB3 and erbB4) was investigated, and also erbB2 because this is the major binding partner for the activated receptors. Associations between the presence of NRG isoform and receptor were then analyzed (Table 2). The majority of tumors were found to coexpress both NRG isoforms and erbB3 and erbB4, either singly or in combination with erbB2. Significant associations were observed between NRG-1 $\alpha$  or NRG-1 $\beta$  and erbB4 and also erbB2/erbB4 coexpression (Table 2). NRG-1 $\beta$  was also significantly associated with erbB2 expression.

To further support the view that NRG can be produced by ovarian carcinoma cells, mRNA expression for a sequence of NRG common to all isoforms was identified using RT-PCR in 20 of 24 (83%) ovarian carcinomas (Fig. 1B) and in 8 of 9 (89%) ovarian cancer cell lines (Fig. 1C).

### Growth Regulation by NRG-1 $\alpha$ and NRG-1 $\beta$ in Ovarian Cancer Cell Lines.

The growth responses to NRG-1 $\alpha$  and NRG-1 $\beta$  were examined in a series of ovarian cancer cell lines. Treatment of 14 cell lines with NRG-1 $\alpha$  at 1 nM for 5 days produced a growth stimulation of  $\geq 15\%$  in 5 of these cell lines (PE01, PE04, PE06, OVCAR-5, and 41M), with increased cell counts varying between 15% and 132% (Fig. 2A).

NRG-1 $\beta$  produced growth stimulations in the same cell lines and also in OVCAR-3 and CAOV3 cells with increased cell counts varying between 60% and 378% (Fig. 2B). The PE01<sup>CDDP</sup> cell line was growth inhibited by NRG-1 $\beta$  to 25% control cell count. The effect of TGF- $\alpha$  at the same concen-



**Fig. 1** Expression of NRG in primary ovarian cancers. *A*, immunohistochemical expression of NRG-1 $\alpha$  and NRG-1 $\beta$  in an ovarian cancer. NRG-1 $\alpha$  was detected using the HRG 76 antibody and NRG-1 $\beta$  using the HRG 102 antibody as described in "Materials and Methods." Note that the epithelial cells within these sections are diaminobenzidine positive (stained brown). The counterstain is hematoxylin (blue). Low ( $\times 100$ ) and high ( $\times 400$ ) magnification images (top and bottom photographs, respectively) are shown. *B*, RT-PCR analysis of NRG mRNA expression in a series of 24 human ovarian cancers. Expression of NRG was detected in 20 of 24 tumors. Amplified PCR products were detected using a 2.5% gel stained with ethidium bromide for UV visualization. *C*, RT-PCR analysis of NRG mRNA expression in a series of nine ovarian cancer cell lines. Lane 1, PE01; Lane 2, PE04; Lane 3, PE06; Lane 4, OVCAR-5; Lane 5, 41M; Lane 6, SW626; Lane 7, 59M; Lane 8, SKOV-3; Lane 9, PE01<sup>CDDP</sup>; Lane 10, water control expression of NRG was detected in eight of nine cell lines, with OVCAR-5 being the only negative in this series.

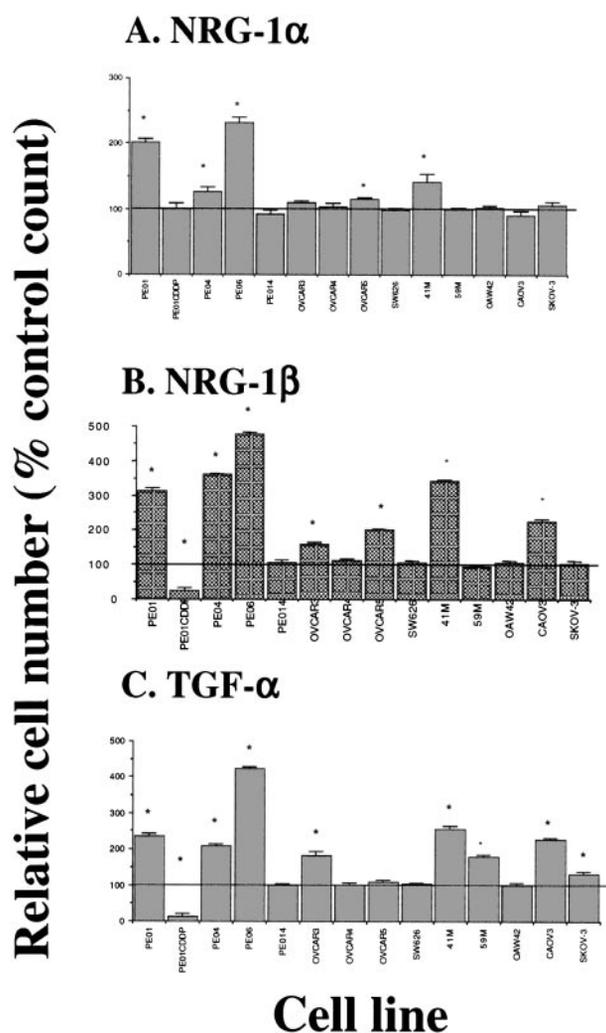
**Table 2** Association of NRG-1 $\alpha$  or NRG-1 $\beta$  expression with erbB2, -3, and -4 receptors in primary ovarian cancer

	NRG-1 $\alpha$ positive (%)	<i>P</i> <sup>a</sup>	NRG-1 $\beta$ positive (%)	<i>P</i>
ErbB2 positive	71.7	0.053	67.9	0.009
ErbB3 positive	74.4	1.00	67.4	0.55
ErbB4 positive	81.1	0.013	71.7	0.042
ErbB2 and erbB4 positive	71.7	0.013	66.0	0.005
ErbB2 and erbB3 positive	58.5	0.40	50.9	0.24

<sup>a</sup> Fisher's exact test.

tration on growth was also investigated in this series, and eight cell lines (PE01, PE04, PE06, OVCAR-3, 41M, 59M, CAOV3, and SKOV-3) were stimulated by between 32% and 122%, with the PE01<sup>CDDP</sup> cell line again showing significant growth inhibition to 13% of control cell number (Fig. 2C).

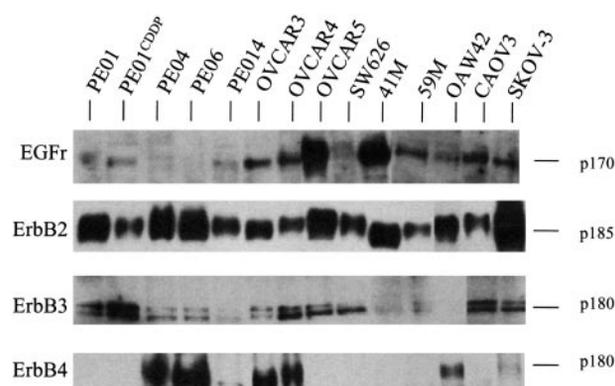
**Magnitude of NRG Growth Response Is Associated with erbB2 in Ovarian Cancer Cell Lines.** The NRGs act via the erbB receptors, and it was of interest to determine whether the growth response was associated with the level of erbB receptor expression. Although NRGs bind to erbB3 and



**Fig. 2** Effect of NRG-1 $\alpha$ , NRG-1 $\beta$ , and TGF- $\alpha$  on the growth of ovarian cancer cell lines. Cells were treated as described in “Materials and Methods” with either NRG-1 $\alpha$  (A), NRG-1 $\beta$  (B), or TGF- $\alpha$  at  $10^{-9}$  M; C) for 5 days. Cells were then counted on a Coulter counter, and the cell number was compared with untreated cells. The SD of the mean of four replicates is indicated. The *asterisk* represents values that are statistically different from control ( $P < 0.05$ , Student’s *t* test). Similar data were obtained in repeat experiments.

erbB4, all potential heterodimerizations have been demonstrated, and so EGF receptor and erbB2 were also measured. The levels of erbB receptors were measured by Western analysis in this series of cell lines (Fig. 3).

The association between NRG-1 $\alpha$  or NRG-1 $\beta$  growth changes with erbB receptor expression is shown in Table 3. Analysis of all 14 cell lines indicates a significant association between NRG-1 $\alpha$  growth stimulation and erbB2 expression ( $P = 0.006$ ; Spearman rank) and near significance for NRG-1 $\beta$  growth stimulation ( $P = 0.079$ ; Spearman rank); however, if the SKOV-3 cell line is excluded, these associations achieve much greater levels of significance with  $P = 0.0015$  for NRG-1 $\alpha$  and  $P = 0.0047$  for NRG-1 $\beta$  (Fig. 4). No significant associations were observed with either NRG or expression levels of any of the other three receptors.



**Fig. 3** Western blots of EGF receptor, erbB2, erbB3, and erbB4 expression in ovarian cancer cell lines. Cell lysates were subjected to Western blot analysis using receptor-specific antibodies as described in the “Materials and Methods.”

**Table 3** Association of NRG-1 $\alpha$  or NRG-1 $\beta$  growth response with EGF receptor, erbB2, -3, and -4 receptor expression in ovarian cancer cell lines

	$P^a$	
	NRG-1 $\alpha^b$	NRG-1 $\beta$
EGF receptor	0.74	0.76
ErbB2	0.006 <sup>c</sup>	0.079 <sup>d</sup>
ErbB3	0.77	0.87
ErbB4	0.21	0.31

<sup>a</sup> Spearman rank correlation.

<sup>b</sup> Expression of erbB receptor was quantified by Western blot analysis and densitometry to give integrated absorbance values. Individual values were then plotted against the growth response value (data shown in Fig. 2) for each cell line. The association between expression and growth response was assessed by use of the Spearman rank test.

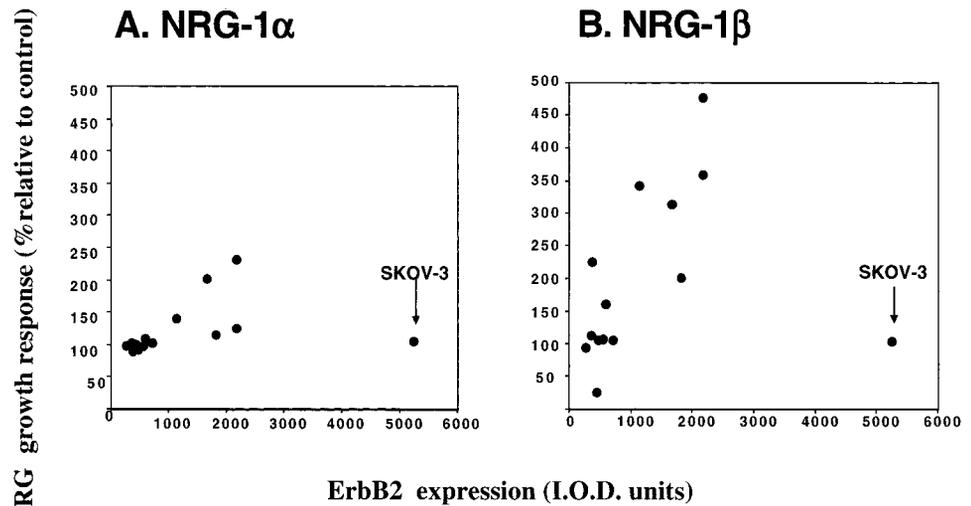
<sup>c</sup> Spearman rank value for all 14 cell lines including SKOV-3. Without SKOV-3,  $P = 0.0015$ .

<sup>d</sup> Spearman rank value for all 14 cell lines including SKOV-3. Without SKOV-3,  $P = 0.0047$ .

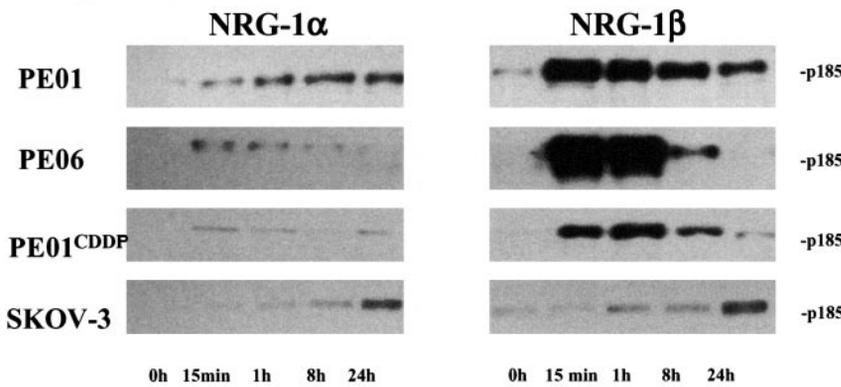
**Signaling Responses to NRG-1 $\alpha$  and NRG-1 $\beta$  in Cell Lines.** Four cell lines were selected for a more detailed analysis of signaling responses to the NRGs. PE01 and PE06 were both growth stimulated by NRG-1 $\alpha$  and NRG-1 $\beta$ , although the latter produced a greater growth response in both cell lines; PE01<sup>CDDP</sup> was growth inhibited by NRG-1 $\beta$  whereas NRG-1 $\alpha$  had no effect on growth and SKOV-3 cells were unaffected by NRG (Fig. 2). Tyrosine-phosphorylated erbB2 and erkK1/2 were monitored. Treatment of PE01 and PE06 cells with NRG-1 $\alpha$  and NRG-1 $\beta$  stimulated phosphorylation of both erbB2 and ERK 1 and ERK2 (Fig. 5), consistent with growth changes. The magnitude of phosphorylation differed with NRG-1 $\beta$  producing greater levels of phosphorylation than NRG-1 $\alpha$ , consistent with a larger growth modulation.

For PE01<sup>CDDP</sup> cells, NRG-1 $\beta$  stimulated phosphorylation of both erbB2 and ERK1/2, this time producing growth inhibition; NRG-1 $\alpha$  produced only minor changes to phosphorylation, consistent with minimal effects on growth. Similarly, SKOV-3 cells, the growth of which was unaffected by

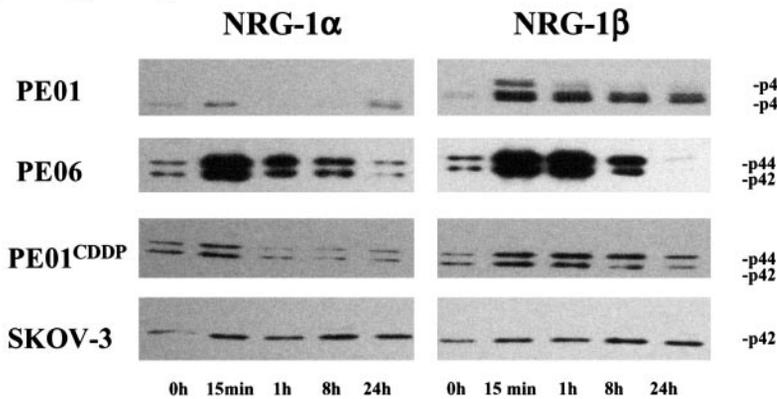
**Fig. 4** Association of NRG-induced growth response with erbB2 expression levels in ovarian cancer cell lines. **A**, NRG-1 $\alpha$  response is associated with erbB2 expression ( $P = 0.006$ , including SKOV-3;  $P = 0.0015$  without SKOV-3, Spearman correlation). **B**, NRG-1 $\beta$  response shows near significance if SKOV-3 is included ( $P = 0.079$ ) and significance ( $P = 0.0047$ ) if SKOV-3 is excluded (Spearman correlation). The erbB2 protein levels were quantified from the Western blot by densitometric analysis using a gel scanner and analyzed by gel analysis software to give integrated absorbance values. These individual values were then plotted against the growth response value (data shown in Fig. 2) for each cell line.



**A. ErbB2 phosphorylation**



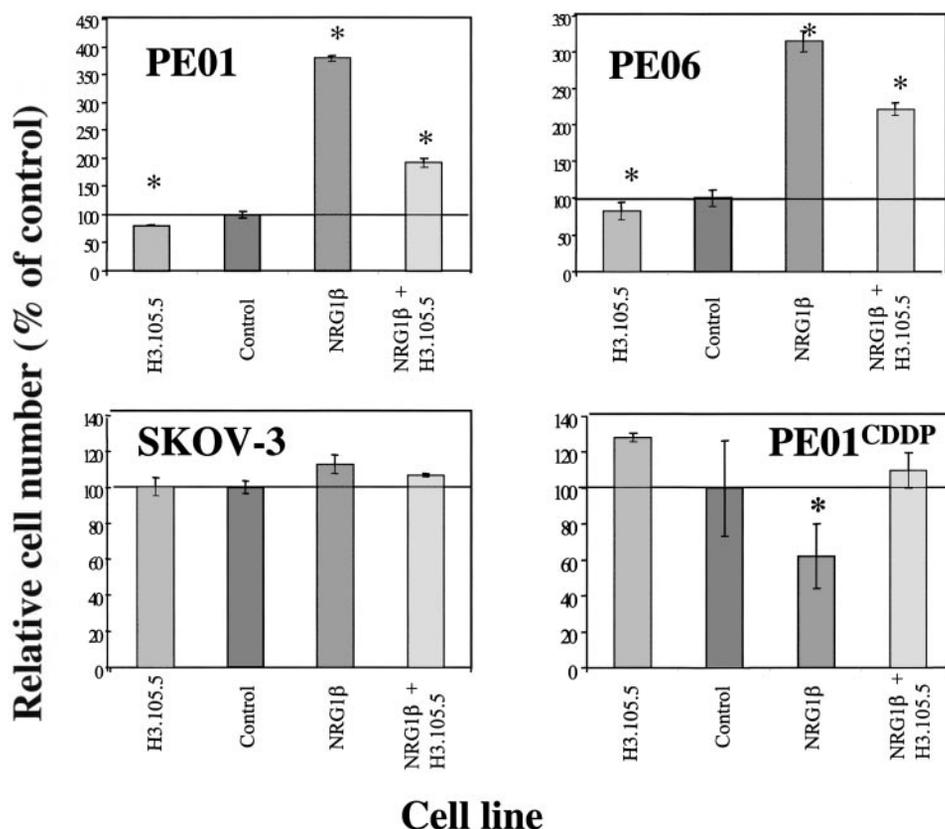
**B. Erk phosphorylation**



**Fig. 5** Phosphorylation of erbB2 and Erk by NRG in ovarian cancer cell lines. A time course of NRG-1 $\alpha$  and NRG-1 $\beta$  ( $10^{-9}$  M) activation is shown. ErbB2 phosphorylation was detected using the antiphosphotyrosine antibody (PY20) and phospho-Erk detected by NEB 9101S, as described in “Materials and Methods”

NRG-1 $\alpha$  or NRG-1 $\beta$ , demonstrated only minor effects on phosphorylation.(Fig. 5). The small increase in phosphorylation of erbB2 at 24 h was also observed in untreated SKOV-3 cells, suggesting that the culture conditions could influence phosphorylation in this cell line.

**Effect of Anti-erbB3 and Anti-erbB4 Antibodies on NRG-induced Growth and Signaling.** We next assessed the relative involvements of erbB3 and erbB4 in NRG-induced growth and signaling. Use of antibodies that bind to either erbB3 (clone H3.105.5; Ref. 28) or erbB4 (H4.72.8; Ref. 28) and block



**Fig. 6** Effect of an anti-erbB3 antibody (H3.105.5) on the growth of ovarian cancer cell lines. Cells were treated as described in “Materials and Methods” with either NRG-1 $\beta$  ( $10^{-9}$  M) alone, H3.105.5 ( $10 \mu\text{g/ml}^{-1}$ ) alone, or a combination of NRG-1 $\beta$  and H3.105.5 for 5 days. Cells were then counted on a Coulter counter, and the cell number was compared with untreated cells. The SD of the mean of four replicates is indicated. The asterisk represents values that are statistically different from control ( $P < 0.05$ , Student’s  $t$  test). Similar data were obtained in a repeat experiment.

the interaction of NRG with the receptor were used to investigate which receptor mediated the growth function. Four cell lines were studied: PE01 and PE06 cells, which were growth stimulated by NRG-1 $\beta$ ; PE01<sup>CDDP</sup>, which was growth inhibited; and SKOV-3, the growth of which was unaffected (Figs. 6 and 7).

Using the anti-erbB3-blocking antibody, the NRG-1 $\beta$  growth effect was partially reversed for PE01 and PE06 cells and completely reversed for PE01<sup>CDDP</sup> (Fig. 7). No change, as expected, was seen in SKOV-3 cells. In the absence of added NRG, reductions of growth were observed for PE01 and PE06, and a small increase was seen with PE01<sup>CDDP</sup> on addition of the antibody, and this would be consistent with reversal of an autocrine level of NRG being produced by these cell lines. These results are consistent with erbB3 mediating the growth response, at least in part.

Using the anti-erbB4-blocking antibody, the NRG-1 $\beta$  growth effects were enhanced rather than blocked in the PE01, PE06, and PE01<sup>CDDP</sup> cells with no change again for SKOV-3 cells (Fig. 7). For PE01 a rather marked increase in growth was seen in the absence of added NRG-1 $\beta$ . These results suggest that erbB4 is not mediating the growth effect and, indeed, appears to be antagonistic.

The effects of herceptin, a humanized antibody that binds

with high affinity to the extracellular domain of erbB2, were assessed against the PE01 and SKOV-3 cell lines. Herceptin, when added alone, had minimal effects on the growth of PE01 cells but reversed the NRG-1 $\beta$  growth stimulation (Fig. 8). A small inhibitory effect of herceptin was seen against SKOV-3 cells both in the absence and in the presence of NRG-1 $\beta$  (Fig. 8).

To investigate the role of the erbB3 receptor in NRG-1 $\beta$ -induced signaling, the effects of the blocking antibody on tyrosine phosphorylation of the erbB2 receptor and Erk 1/2 were studied in PE01 cells. Treatment of PE01 cells with NRG-1 $\beta$  markedly increased tyrosine phosphorylation of both erbB2 (Fig. 9A) and phosphorylation of both p44 (ERK1) and p42 (ERK2; Fig. 9B). This was completely reversed by coinubation of the anti-erbB3 antibody, demonstrating the involvement of erbB3 in NRG-1 $\beta$  signaling. In contrast, coinubation with the anti-erbB4 antibody produced minimal, if any, changes on signaling.

## DISCUSSION

This is the first detailed description of the expression of NRG isoforms in primary ovarian cancer. Both NRG-1 $\alpha$  and NRG-1 $\beta$  were found in over 75% of ovarian cancers, with

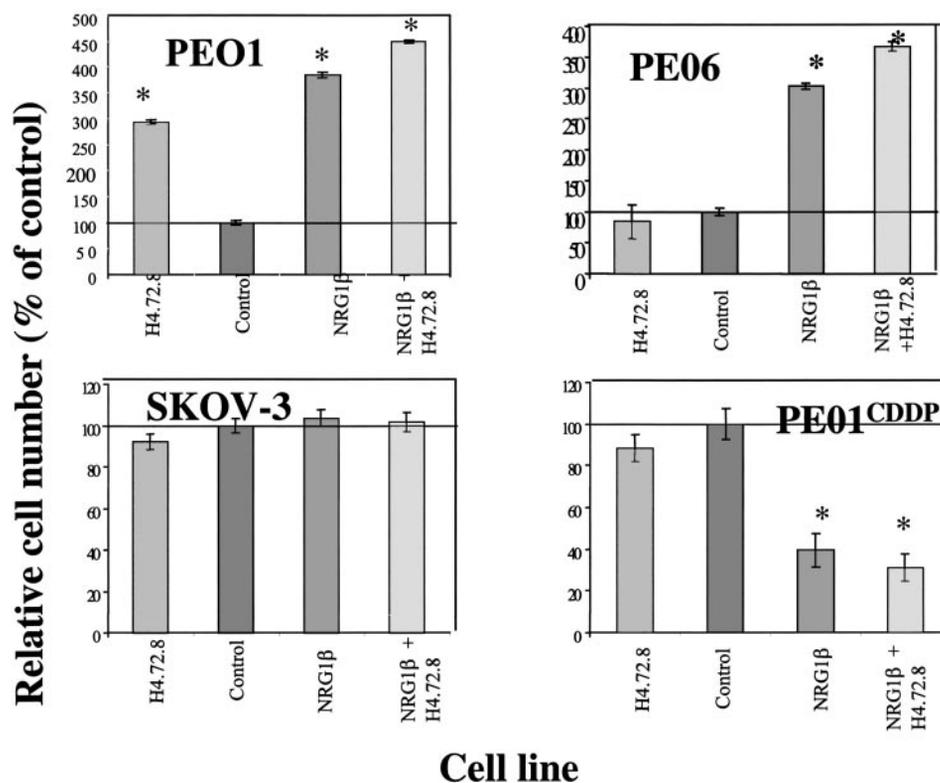


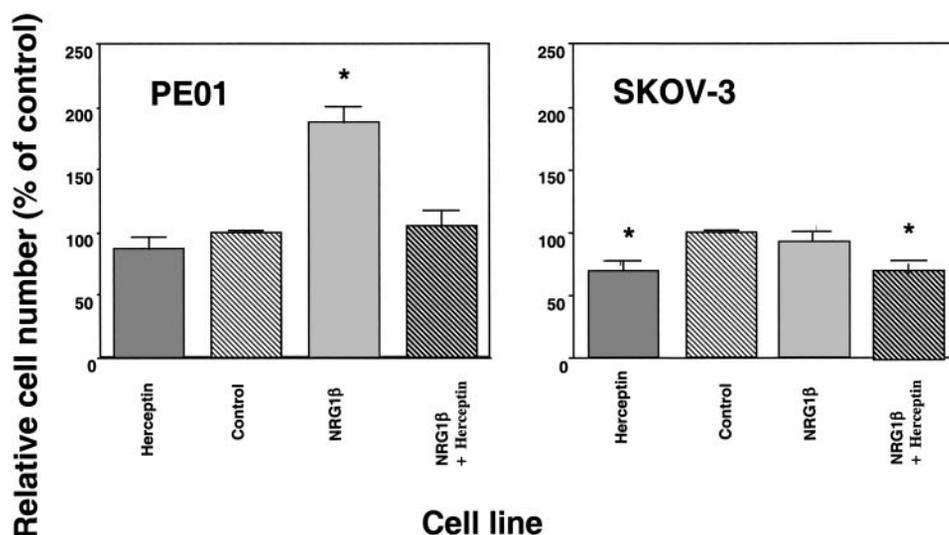
Fig. 7 Effect of an anti-erbB4 antibody (H4.72.8) on the growth of ovarian cancer cell lines. Cells were treated as described in “Materials and Methods” with either NRG-1 $\beta$  ( $10^{-9}$  M) alone, H4.72.8 ( $10 \mu\text{g}/\text{ml}^{-1}$ ) alone, or a combination of NRG-1 $\beta$  and H4.72.8 for 5 days. Cells were then counted on a Coulter counter, and the cell number was compared with untreated cells. The SD of the mean of four replicates is indicated. The asterisk represents values that are statistically different from control ( $P < 0.05$ , Student's  $t$  test). Similar data were obtained in a repeat experiment.

expression detected in carcinoma cells and not the stroma. RT-PCR analysis provided similar data with 83% of ovarian cancers expressing NRG mRNA. Serous tumors tended to have a higher level of expression than tumors of endometrioid histology and were associated with a poorer prognosis. Expression of NRG receptors, namely erbB3 and erbB4, are commonly found in this disease (17–20), and analysis of coexpression patterns indicated significant associations between NRG growth response and the presence of erbB4 and/or erbB2.

The potential for autocrine and paracrine growth regulation was, therefore, present, and we next sought to investigate the functionality of exogenously added NRGs in a large panel of ovarian cancer cell lines. NRG-1 $\beta$  produced growth stimulation in 7 of 14 of these lines and was generally more potent than TGF- $\alpha$  on an equimolar basis. Variable results have been reported with respect to the growth effects of NRG-1 $\beta$  in ovarian cancer cell lines (21–24). Pegues *et al.* (21) found no growth stimulation (in monolayer and soft agar) in five of five ovarian cancer cell lines, including the SKOV-3 and OVCAR-3 lines. Our data are consistent with a lack of effect with SKOV-3 cells but we did observe stimulation with OVCAR-3 cells. Xu *et al.* (22) reported growth inhibition in agar with SKOV-3 cells and stimulation with five other cell lines, whereas Lewis *et al.* (23) showed stimulation of anchorage-independent growth of SKOV-3 but not CaOv3 cells. Finally, Aguilar *et al.* (24), using a variety of assays, reported stimulatory effects in three of three

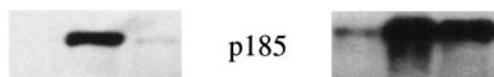
ovarian cancer cell lines, but these effects were assay dependent. Some of the variability between these results is likely to be due to different assay endpoints, however, the level of serum has been proposed to profoundly influence the NRG response (23). Our use of charcoal-stripped serum may allow some of these responses to be more easily seen. We are aware of only a single study that has previously explored the effects of NRG-1 $\alpha$  in ovarian cancer cells (25). Campiglio *et al.* (25) demonstrated increased  $^3\text{H}$ -thymidine incorporation in 2 of 4 ovarian cancer cell lines, and our results demonstrating growth stimulation in 5 of 14 cell lines are consistent with this. Our study has, therefore, not only extended these previous observations indicating that NRGs can stimulate growth but also allows direct comparison of these two isoforms and indicates that NRG-1 $\beta$  is more potent than NRG-1 $\alpha$  within this panel of cell lines.

An important observation was made by Xu *et al.* (22), who suggested that the outcome of NRG-induced activation of anchorage-independent growth was dependent on the relative levels of erbB2 and erbB3. Within that study, they suggested overexpression of erbB2 was related to growth inhibition whereas lower levels were related to stimulation. This was based on SKOV-3 cells being growth inhibited by NRG-1 $\beta$  whereas five other cell lines were growth stimulated. In the present study, we observed that for cell lines other than SKOV-3 there was a highly significant association between level of erbB2 expression and magnitude of growth stimulation produced by both NRG-1 $\alpha$  and NRG-1 $\beta$ , suggesting

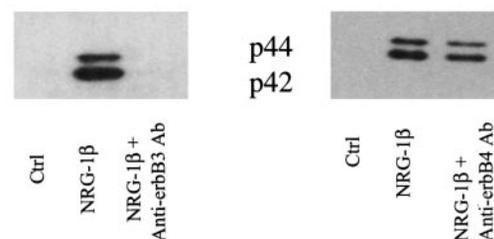


**Fig. 8** Effect of herceptin on the growth of the PE01 and SKOV-3 ovarian cancer cell lines. Cells were treated as described in “Materials and Methods” with either NRG-1 $\beta$  ( $10^{-9}$  M) alone, herceptin ( $1 \mu\text{M}$ ) alone, or a combination of NRG-1 $\beta$  and herceptin for 5 days. Cells were then counted on a Coulter counter, and the cell number was compared with untreated cells. The SD of the mean of four replicates is indicated. The *asterisk* represents values that are statistically different from control ( $P < 0.05$ , Student’s *t* test). Similar data were obtained in a repeat experiment.

### A. ErbB2 phosphorylation



### B. Erk phosphorylation



**Fig. 9** Effect of an anti-erbB3 (H3.105.5) or anti-erbB4 antibody (H4.72.8) on erbB2 p185 tyrosine phosphorylation (A; detected using an anti-phosphotyrosine antibody) and p42 and p44 ERK phosphorylation (B; using an antiphosphoERK antibody) in PE01 cells after NRG-1 $\beta$  stimulation. Cells were treated as described in “Materials and Methods” with either NRG-1 $\beta$  ( $10^{-9}$  M) alone or a combination of NRG-1 $\beta$  and antibody for 5 days. Cell lysates were then prepared for Western blot analysis.

that the level of erbB2 was the critical determinant. Clearly, either erbB3 or erbB4 must mediate the NRG binding, but the association of expression of erbB2 with magnitude of response argues not only for the process of heterodimerization of either erbB3 or erbB4 with erbB2 but also that signaling via erbB2 is limiting. Further support for the involvement of erbB2 was provided by Aguilar *et al.* (24), who transfected erbB2 into several ovarian cancer cell lines and demonstrated an increased anchorage-independent growth stimulation with NRG-1 $\beta$ . In our study, SKOV-3 cells were the clear exception and, despite having extremely high levels of erbB2, were

unresponsive to the NRGs. It is feasible that this is due in some way to the exceptionally high level of erbB2 receptors, perhaps causing ligand-independent phosphorylation or due to the presence of autocrine regulation.

Direct evidence for erbB3 mediating the growth modulatory effects of the NRGs was then obtained by both blockade of the growth modulations and inhibition of signaling by the use of a blocking antibody. These data argue strongly for the involvement of erbB3 in mediating this potent growth effect. In contrast, erbB4 blockade enhanced the effects of NRG-1 $\beta$ , suggesting that signaling via erbB4 antagonizes the growth stimulatory effect of NRG.

These data strongly support the possibility of autocrine growth regulation by NRG in this disease. First, in primary tumors and in cell lines, both the ligand and its receptors are widely expressed. Second, exogenously added ligand produces growth stimulation. This stimulation can be blocked by use of an antibody that binds to the erbB3 receptor, indicating involvement of erbB3 in the autocrine loop. Finally, in the absence of exogenously added ligand, the blocking antibody reduced the growth of both PE01 and PE06 cells and increased the growth of PE01<sup>CD<sub>133</sub></sup> cells, consistent with the presence of endogenous NRG, the presence of which was confirmed by RT-PCR in these lines.

If NRG is promoting the growth of this disease, it may be a useful therapeutic target. Because both our results and those of others indicate dimerization with erbB2 is likely to be involved in mediating the growth regulatory effects, removal of erbB2 from the cell surface with the recombinant humanized anti-erbB2 antibody herceptin (trastuzumab) may prove a useful therapeutic strategy in blocking NRG-driven growth. This agent has already shown useful activity in erbB2-overexpressing breast cancers (29). Some support for this view was obtained with the PE01 cell line.

In conclusion, this is the first systematic analysis of NRG expression in ovarian cancer. We have demonstrated that NRG expression is found in the majority of ovarian cancers together with their receptors. Both NRG-1 $\alpha$  and NRG-1 $\beta$  promote growth of ovarian cancer cells and, for the latter ligand, erbB3 appears to mediate growth. The levels of erbB2 expression, however, determine the magnitude of these effects and it seems likely that autocrine growth regulation via NRG/erbB3 can occur *in vivo*.

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## Neuregulin Expression, Function, and Signaling in Human Ovarian Cancer Cells

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