

# Mesenchymal Transformation in Epithelial Ovarian Tumor Cells Expressing Epidermal Growth Factor Receptor Variant III

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Overexpression of the epidermal growth factor (EGF) receptor occurs frequently in ovarian cancer and is associated with poor patient prognosis. A constitutively active mutant EGF receptor termed variant III (EGFRvIII) has been detected at a high frequency in many human tumors, including those of the ovary. To identify the consequences of EGFRvIII expression in ovarian tumor cells, we introduced EGFRvIII into the epithelial ovarian cancer cell line (OVCA 433). The EGFRvIII-transfected cells displayed a dissociated, motile phenotype and fibroblastic morphology. The EGFRvIII-dependent phenotype was comparable to that observed in EGF-stimulated parental OVCA 433 cultures and required the catalytic activity of the mutant receptor. Disruption of adherens and desmosomal junctions in EGFRvIII expressing cells was evident by immunofluorescent detection of specific junctional components. In addition, Western blot analysis confirmed decreased levels of cellular plakoglobin and  $\beta$ -catenin in EGFRvIII-expressing cells, and E-cadherin protein and mRNA were nearly absent. The loss of E-cadherin was accompanied by decreased expression of additional ovarian epithelial markers, including keratins 7, 8, and 18 and mucins 1 and 4. In contrast, the mesenchymal markers N-cadherin and vimentin were elevated in EGFRvIII expressing cells. Overall, the switch in cadherins from E-cadherin to N-cadherin, coupled with gain of vimentin expression and loss of the epithelial keratins and mucins typically expressed in well-differentiated epithelial ovarian carcinomas, are consistent with transition to a mesenchymal phenotype as an outcome of EGFRvIII expression. These findings suggest that EGFRvIII expression may regulate phenotypic plasticity in ovarian cancer and thereby contribute to more aggressive disease. © 2006 Wiley-Liss, Inc.

**Key words:** epidermal growth factor receptor; EGFR; EGFRvIII; signal transduction; ovarian cancer; adherens junctions; epithelial-mesenchymal transition

## INTRODUCTION

Ovarian carcinoma is the leading cause of death from gynecologic malignancy, resulting in approximately 16210 deaths in 2005 [1], with epithelial tumors accounting for ~90% of ovarian malignancies [2]. In the adult female, the normal ovarian epithelium is a single cell layer separated by a subepithelial basement membrane from an underlying stroma, the tunica albuginea, which comprises dense collagenous connective tissue [2]. The mesodermally derived ovarian surface epithelium (OSE) is a simple epithelium that displays epithelial and mesenchymal characteristics and contains both keratin and vimentin intermediate filaments. In tissue culture, normal ovarian epithelial cells express epithelial markers including keratins 7, 8, and 18, desmoplakin and mucin 1, as well as mesenchymal markers such as vimentin and neural (N)-cadherin [2,4]. Ovarian epithelial cells exhibit phenotypic plasticity; reversible modulation of ovarian epithelium to a fibroblastic form occurs

during postovulatory repair and is also observed in ovarian tumor progression [2,3].

In the early stages of neoplastic transformation, OSE undergoes Mullerian differentiation so that new epithelial features appear, while the mesenchymal characteristics of OSE diminish [2]. The new epithelial features include altered cell shape [5], appearance of epithelial (E)-cadherin, formation of junctional complexes [2,6,7], expression of epithelial membrane antigens, and increased production of

Abbreviations: OSE, ovarian surface epithelium; EGF, epidermal growth factor; EGFRvIII, epidermal growth factor receptor variant III; PBS, phosphate buffered saline; EMT, epithelial to mesenchymal transformation.

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secretory products including mucins (mucin1-4) and CA125 [2,5]. At later stages of tumor progression, some of these specialized epithelial characteristics, such as E-cadherin expression, may diminish as cells de-differentiate [6–8] and acquire mesenchymal properties associated with a more aggressive phenotype.

The mechanisms regulating phenotypic transitions in ovarian cancer are unknown; however, reversible modulation of ovarian epithelial cells during postovulatory repair has been linked to expression and activity of the epidermal growth factor (EGF) receptor [2,3]. Overexpression of the EGF receptor and its ligands is often detected in ovarian tumors [9–13] and is associated with a less favorable prognosis. Aberrant EGF receptor activation may play a critical role in ovarian tumor progression and metastasis through control of proliferation [14–17], regulation of cell:cell and cell:matrix interactions [18–20], stimulation of cell migration [18,21], and induction of many matrix degrading proteases including those belonging to the plasminogen activator and matrix metalloproteinase families [20]. Soluble ligand-binding variants of the EGF receptor (extracellular domain) are also linked to disease progression in ovarian cancer [13]. Although the role of these soluble receptors in ovarian pathophysiology is unclear, it has been suggested that truncated ectodomain variants may modulate EGF receptor autophosphorylation [13]. Importantly, decreased expression of EGF receptor in ovarian carcinoma cells in culture reduces their malignant character [22,23]. Together, these findings suggest that EGF receptor activation is a factor in numerous aspects of ovarian cancer biology.

Epidermal growth factor receptor can be activated by ligand binding or receptor mutation. Naturally occurring EGF receptor mutations occur in human tumors. Of particular interest is a mutant EGF receptor designated  $\Delta$ EGFR, de2-7 EGFR or EGFR variant III (EGFRvIII). The EGFRvIII is a 145-kDa EGF receptor with a deletion in the extracellular domain of the receptor corresponding to nucleotides 275–1075 (exons 2 through 7) of the EGF receptor cDNA [24,25]. This mutant EGF receptor has been frequently detected in a variety of human tumors including glioblastoma, breast, lung, prostate, and ovarian cancers [26–30]. This mutant receptor does not bind EGF [31]; nevertheless, it is constitutively active as detected by receptor dimerization [31], autophosphorylation [32], and activation of key signal transduction cascades [28,33]. EGFRvIII is functionally active without ligand in models of tumorigenesis; it stimulates DNA synthesis, transformation of fibroblasts, and potentiates tumor growth in nude mice [28,33]. In addition, expression of EGFRvIII in a glioblastoma or small cell lung cancer cell line renders the cells more invasive [34,35].

The potential impact of EGFRvIII expression on the development or progression of ovarian cancer is currently unknown. In this study, we stably expressed EGFRvIII in an epithelial ovarian carcinoma cell line (OVCA 433). We found that EGFRvIII expression resulted in a dispersed phenotype characterized by increased cell migration, dissolution of adherens and desmosomal junctions, and down-regulation of certain junctional proteins. Interestingly, protein and mRNA levels of the epithelial marker E-cadherin were significantly decreased in the EGFRvIII-expressing cells when compared to ligand-stimulated parental or vector control cells. The decrease in E-cadherin was accompanied by an increase in N-cadherin expression. Other epithelial markers were diminished in EGFRvIII-expressing cells including keratins 7, 8, and 18, and mucins 1 and 4, whereas vimentin expression was elevated. These results suggest that the molecular changes associated with EGFRvIII expression may drive epithelial-mesenchymal transition, and contribute to metastatic dissemination of ovarian cancer.

## MATERIALS AND METHODS

### Cell Culture and Treatment

Ovarian carcinoma cell lines OVCA 433, and OVCA 429 were generously provided by Dr. Robert Bast, Jr., M. D. Anderson Cancer Center, Houston, TX and grown as described previously [18,19,36]. Briefly, cells were grown in minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.5 units/mL penicillin, 0.5  $\mu$ g/mL streptomycin (this medium is referred to later as complete growth medium). Cells were maintained at 37°C under 5% carbon dioxide. The OVCA 433 cell line was selected for transfection based on low to moderate expression of endogenous wild-type EGF receptor compared to other OVCA lines, as determined by Western blot analysis and evidence of intact EGF-dependent signaling pathways (data not shown). The EGFRvIII construct was a generous gift of Dr. David Moscatello, Thomas Jefferson University, Philadelphia, PA. OVCA 433 cells were cotransfected with a vector containing a neomycin resistance gene and either the EGFRvIII construct in PLTR2 vector or the PLTR2 vector at a 1:10 ratio by calcium phosphate method. Clones were selected in presence of 300  $\mu$ g/mL G418 (Gibco<sup>Q1</sup>/BRL). Ten EGFRvIII-transfected clones were isolated from two independent transfections. All clones displayed the same phenotype, and were similar. Two EGFRvIII-transfected clones, designated EGFRvIII A1 and EGFRvIII A2, were selected for extensive characterization. For experiments involving EGF (Biomedical Technologies, Stoughton, MA), OVCA 433 cell lines were placed into MEM containing 0.1% (w/v) bovine serum albumin (BSA) for 24 h prior to growth factor

addition, as described previously [18,19]. Treatment with the EGF receptor catalytic inhibitor AG1478 (CalBiochem, LaJolla CA) was conducted in complete growth medium unless otherwise noted in the figure legends.

#### Immunofluorescence

An antibody against the novel epitope in EGFRvIII (Ab 1825) was raised in chickens by Aves Laboratory (Tigard, OR) as described elsewhere [25] with the following modifications: the anti-peptide antibody was produced for the peptide LEEKKGNYVVDHC after adding an amino caproic acid just upstream of the C-terminal cysteine, which was used for conjugation to keyhole limpet hemocyanin. Antibodies used in immunofluorescence included mouse anti-E-cadherin antibody #C20820 (Transduction Laboratory, Lexington, KY), mouse anti- $\beta$ -catenin antibody #MAB2081 (Chemicon, Temecula, CA), and a chicken anti-plakoglobin antibody (generous gift of Dr. K. Green, Northwestern University, Chicago, IL). Cells were seeded in a Lab Tek II chamber slide system (Nalge Nunc Int., Naperville, IL). For detection of EGFRvIII, cells were fixed for 7 min at room temperature in freshly prepared 3.7% (w/v) paraformaldehyde in phosphate buffered saline (PBS: 0.137 M sodium chloride, 27 mM potassium chloride, 43 mM dibasic sodium phosphate, 15 mM monobasic potassium phosphate). Cells were incubated in blocking buffer (10% nonfat dry milk in PBS) for 1 h at 37°C, washed three times with PBS, incubated with chicken anti-EGFRvIII antibody 1825 at 1:800 dilution prepared in the blocking solution, for 1 h at 37°C, washed with PBS, incubated with anti-chicken-fluoresceinated antibody (1:900 dilution in blocking buffer) for 20 min at 37°C, and mounted with Vectorshield mounting media. For all other antibodies, cells were fixed for 2 min in ice-cold, dehydrated methanol. For detection of E-cadherin,  $\beta$ -catenin, and plakoglobin, cells were washed with PBS containing 0.8 mM magnesium sulfate, and 0.18 mM calcium chloride. For detection of  $\beta$ -catenin only, cells were permeabilized by incubating in cold 0.1% Triton X-100 in PBS containing calcium and magnesium on ice for 5 min, then cells were washed three times with PBS containing calcium and magnesium. Cells were incubated in blocking buffer (3% BSA in PBS containing calcium and magnesium) and processed as described above with primary antibodies directed against E-cadherin,  $\beta$ -catenin, and plakoglobin at 1:100 dilution in blocking buffer. Cells were then incubated for 1 h at 37°C with secondary antibodies (anti-mouse-FITC conjugated antibody #1034-02, Southern Biotechnology Associates, Birmingham, AL or anti-chicken-FITC conjugated antibody #F-1005, Aves Labs) at 1:500 dilution prior to washing and mounting. Slides were examined with an Olympus BH2-RFCA microscope (Melville, NY) and

images were obtained with an Omegafire digital camera (Optronix, Goleta, CA).

#### Western Blot Analysis

Control and treated cells were washed with ice-cold PBS and harvested in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 5 mM EDTA, 0.1 mM dithiothreitol and 1 mM PMSF). Ten micrograms of total cell lysate was resolved by electrophoresis through 10% SDS-polyacrylamide, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA) and probed with the indicated antibodies. These included antibody for intracellular domain of EGFR (sc-03, Santa Cruz, Santa Cruz, CA), and anti-phosphorylated ERK (#9101 Cell Signaling, Beverly, MA) at 1:1000 dilutions, a cocktail of antibodies against phospho-EGFR (#2237, 2234, 2235, 2231 Cell Signaling) at 1:1000 each, anti-E-cadherin (Ab H108, Santa Cruz), anti-plakoglobin (AbH80, Santa Cruz), anti- $\beta$ -catenin (E5, Santa Cruz), and anti-desmoglein (Transduction Labs) at 1:500 dilutions; anti-N-cadherin (Zymed Laboratories, Inc., San Francisco, CA) at 1:250 dilution; and anti-vimentin, anti-mucin-1 (Chemicon) and anti-keratins 7, 8, and 18 (#K0199-07, K0199-10, and K0199-21, respectively, US Biological, Swampscott, MA) at 1:100 dilution. Secondary antibodies were obtained from Promega (Madison, WI). Quantitation was performed by direct luminescence detection with SuperSignal Pico chemiluminescent substrate (Pierce, Rockford, IL) and the Kodak Image Station 440 ([NEN Life Science Products](#)<sup>Q2</sup>). Quantitation was performed on samples obtained from three independent experiments and values represent the mean  $\pm$  standard deviation.

#### PCR Detection of mRNA

RNA was extracted from cells growing in 10 cm tissue culture plates with Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) as recommended by the vendor. Reagents for PCR were obtained from Invitrogen Life Technologies. To generate first strand cDNA, 250 ng of random primers was annealed to 2  $\mu$ g of total RNA in a 12  $\mu$ L reaction, which was heated to 70°C for 10 min, then quick chilled on ice. Then 10 nmol of each dNTP, 200 nmol DTT, 30 U RNase inhibitor, and 5 $\times$  first strand buffer were added, so the total volume was 19  $\mu$ L. The tubes were incubated at 42°C for 2 min, followed by addition of 200 U of SuperScript II RNase H<sup>-</sup> reverse transcriptase and incubation at 25°C for 10 min, at 42°C for 50 min, and at 70°C for 15 min. The PCR reaction mixture contained 2  $\mu$ L of cDNA, 25  $\mu$ L of Taq PCR master mix, 5 pmol of each primer and the volume was brought to 50  $\mu$ L. The E-cadherin primers were 5'GGGTGACTACAAAATCAATC3' and 5'GGGGGCAGTAAGGGCTCTTT3' [37]. Thermal cycling started with an initial denaturation step at 94°C for 4 min and ended with a final extension step at 72°C for 7

min. For E-cadherin primers, thermal cycling consisted of 40 cycles of 94°C for 60 s, 60°C for 90 s, and 72°C for 120 s. PCR amplifications were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Inc. Watertown, MA). PCR samples were then electrophoresed through 2% agarose gel and visualized by ethidium bromide staining.

For real-time PCR, the first strand cDNA was generated as described above. The real-time PCR was performed with TaqMan universal conditions (Applied Biosystems, Foster City, CA), in which 900 nM of each primer and 250 nM of the probe were used for E-cadherin. The E-cadherin primers were 5'AGGTGACAGAGCCTCTGGATAGA3' and 5'CATTCCCGTTGGATGACACA3', and the E-cadherin probe was FAM-CGCATTGCCACATACACTC-TCTTC-TAMRA. In addition, real-time PCR was performed for GAPDH with the Applied Biosystems GAPDH control reagent kit according to the manufacturer's protocol with 250 nM of each primer and 100 nM of the probe. Real-time PCR reactions were carried out on 200 ng of cDNA in a total volume of 45  $\mu$ L containing TaqMan 2 $\times$  master mix with an ABI prism 7000 sequence detection system. Evaluation of amplification efficiency for E-cadherin and GAPDH was performed according to Applied Biosystems recommendations, and the efficiency of the two amplification reactions was found to be equivalent. The transcripts were quantified with the  $\Delta\Delta C_t$  method according to Applied Biosystems recommendations with GAPDH as the normalizer. For quantitation of E-cadherin, EGFRvIII A1 was used as the calibrator.

#### Transplantation

Cells were resuspended at  $10^7$ /mL in culture medium without fetal bovine serum, then mixed with an equal volume of Matrigel. The mixture was chilled to prevent polymerization of the Matrigel. One milliliter of the mixture was injected subcutaneously in the flank region of each of several nude mice. After 2 mo, animals were killed by CO<sub>2</sub> inhalation and masses were identified grossly at 1/4 injection sites for vector control cells, 2/4 injection sites for clone A1 of EGFRvIII-expressing cells, and 1/3 injection sites for clone A2 of EGFRvIII-expressing cells. Each mass was approximately 1  $\times$  0.5 mm in size; masses were translucent, with a waxy texture.

#### Immunohistochemistry

Tumor sections were stained for cytokeratin, vimentin, E-cadherin, and N-cadherin. After deceraction (removal of wax), antigen retrieval was performed with Dako Target Retrieval Solution as suggested by the manufacturer (Dako, Carpinteria, CA). Staining was performed with a Dako Autostainer. The primary antibodies included mouse monoclonals raised against human epidermal keratin

(Clones AE1 and AE3, Dako, a fragment of human E-cadherin (Clone 36, BD Biosciences, Franklin Lakes, NJ), and N-cadherin (Clone 3B9, Zymed) and a rabbit polyclonal anti-vimentin antibody (Biomed, Foster City, CA). Briefly, endogenous peroxidase activity was blocked for 5 min with Dako peroxidase block, protein block (Dako was applied for 5 min or PowerBlock (BioGenex, San Ramon, CA) for 10 min, slides were incubated with primary antibody diluted 1:50 (anti-keratin), 1:100 (anti-N-cadherin, anti-E-cadherin), or 1:750 (anti-vimentin) for 30 min and with appropriate LSAB2 (Dako or Vectastain Elite (Vector, Burlingame, CA) secondary reagents for 30 min. Diaminobenzidine was used for color development, and sections were counterstained with hematoxylin, dehydrated, and mounted.

#### Statistical Analysis

For all analyses, differences between each two cell lines being compared were evaluated with a Welch's two sample t-test. *P*-values below 0.05 were considered statistically significant.

## RESULTS

### EGFRvIII Expression in OVCA 433 Cells Promotes a Dissociated Cell Phenotype

EGF receptor termed variant III was stably expressed in an ovarian tumor cell line (OVCA 433), as described in Materials and Methods. Expression of EGFRvIII in transfected cells was confirmed by immunofluorescence microscopy, with an antibody raised against the unique epitope formed by the exon 2-7 deletion characteristic of this EGF receptor mutation. Staining for EGFRvIII was observed in the transfected cell lines EGFRvIII A1 (A1) and EGFRvIII A2 (A2), while the parental OVCA 433 (P) and control transfected (V) cells were not immunoreactive with the EGFRvIII antibody (Figure 1A). Furthermore, expression of EGFRvIII was evident in the transfected cells as detected by an antibody recognizing the intracellular domain of EGFR and EGFRvIII (Figure 1B). In Figure 2A, the phenotype of two EGFRvIII transfected clones is compared to that of ligand-activated parental OVCA 433 cells. In the absence of EGF, OVCA 433 (Figure 2A) and OVCA 429 [19] colonies displayed an epithelioid morphology and cells were tightly associated. Activation of EGF receptor by EGF promoted a migratory response as detected by increased colony dispersion (Figure 2A) and enhanced *in vitro* wound closure (data not shown) in both cell lines. The EGFRvIII expressing clones EGFRvIII A1 and EGFRvIII A2, in the absence of exogenous ligand, recapitulated the phenotype of EGF-stimulated parental OVCA 433 cells (Figure 2A). Importantly, the phenotype was reversed when cells were treated with AG1478 (Figure 2B), a highly selective inhibitor of the

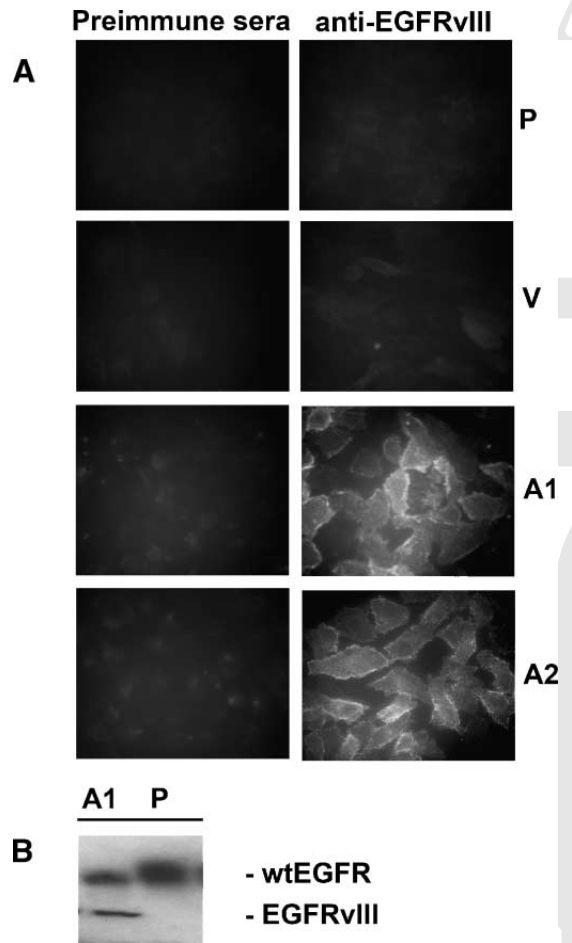


Figure 1. Detection of EGFRvIII in stably-transfected cells. (A) Detection by immunofluorescence microscopy. Cells were fixed and stained with either preimmune sera or with anti-EGFRvIII Ab 1825 antibody as described in Materials and Methods. (B) Detection by Western blot analysis. Total cell lysate (35 µg protein) was loaded per well. An antibody to the intracellular domain of EGFR and EGFRvIII was used to detect both wild-type and EGFRvIII expression. EGFRvIII was detected in EGFRvIII-expressing cells only. P, parental OVCA 433 cell line; V, vector-transfected cell line; A1, EGFRvIII clone A1; and A2, EGFRvIII clone A2.

catalytic activity of both the wild-type EGF receptor and EGFRvIII [38]. Treatment of EGFRvIII-expressing clones with AG1478 for 6 d inhibited the tyrosine phosphorylation of EGFRvIII and wild-type EGFR in addition to reducing ERK phosphorylation (Figure 2C). Withdrawal of inhibitor from the growth medium restored phosphorylation of EGFR and ERK (Figure 2C). EGFRvIII-expressing clones resumed a phenotype resembling the parental cells with extended AG1478 treatment, (Figure 2B), but returned to a dispersed phenotype after removal of the inhibitor from the culture medium (Figure 2B, right panels). This finding indicates that the migratory phenotype of EGFRvIII-expressing cells is dependent on the tyrosine kinase activity of transfected EGFRvIII.

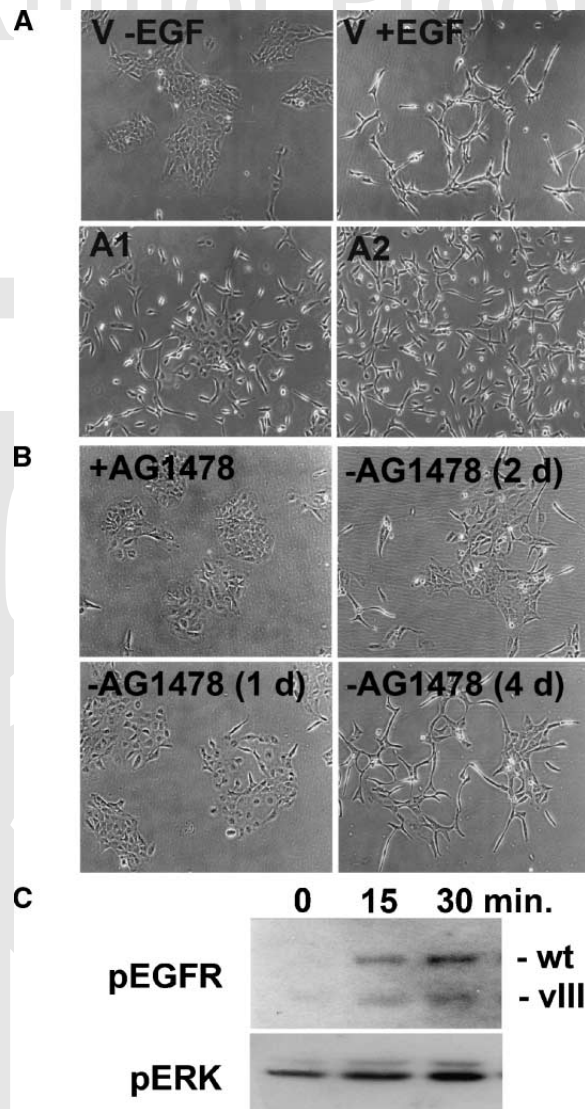


Figure 2. Morphology of EGFRvIII-expressing epithelial ovarian tumor cells. (A) Vector-transfected cells (V) were treated without (V - EGF) or with (V + EGF) 25 nM EGF for 24 h, which promoted colony dispersion. Clones EGFRvIII A1 (A1) and EGFRvIII A2 (A2) both display altered morphology and colony dispersion in the absence of EGF. (B) Reversible modulation of the dispersed phenotype in EGFRvIII expressing cells. EGFRvIII A1 cells were treated with the EGF receptor tyrosine kinase inhibitor AG1478 (2 µM) in growth medium for 21 d (upper left panel), resulting in reversal of the dispersed phenotype. Removal of AG1478 from the growth medium (1, 2, and 4 d) leads to restoration of the scattering response in the absence of EGF. (C) EGFRvIII A1 cells were treated with AG1478 (2 µM) for 6 d, then the medium was replaced without inhibitor and cell lysates collected after 0, 15, and 30 min. Whole cell lysates (10 µg protein) were fractionated by PAGE and phosphorylated EGFR receptor (wild-type and EGFRvIII), and phosphorylated ERK were detected by immunoblot analysis. The figure is representative of three independent experiments.

#### EGFRvIII Expression Disrupts Cell: Cell Junction Integrity

We previously reported that disruption of cell:cell contacts is associated with EGF-stimulated colony dispersion in ovarian tumor cell lines [19]. In a number of carcinomas, loss of E-cadherin expression

has been associated with disease progression and metastasis [39,40]; however, in ovarian epithelial carcinomas, a complex pattern of E-cadherin expression is observed. E-cadherin expression accompanies the onset of tumorigenesis [2], yet at later stages of tumor progression, E-cadherin expression may diminish [6–8]. In the present study, immunofluorescence analysis of adherens junction proteins (E-cadherin and  $\beta$ -catenin) and a desmosomal protein (plakoglobin/ $\gamma$ -catenin) revealed loss of functional junctions in EGFRvIII expressing cells, as detected by protein loss or redistribution of protein from the membrane to the cytoplasm (Figure 3). In addition, there was an apparent decrease in total E-cadherin and plakoglobin protein in EGFRvIII-expressing cells (Figure 3).

Western blot analysis demonstrated selective down regulation of junctional proteins in response to EGF treatment or EGFRvIII expression (Figure 4A). Expression of EGFRvIII significantly decreased E-cadherin ( $P=0.001$ ), plakoglobin ( $P=0.007$ ), and  $\beta$  catenin ( $P=0.020$ ) protein levels (Figure 4B); whereas expression of the desmosomal proteins desmoglein (Figure 4A and B) and desmocollin (data not shown) were not altered. E-cadherin protein was essentially undetectable in EGFRvIII-expressing cells

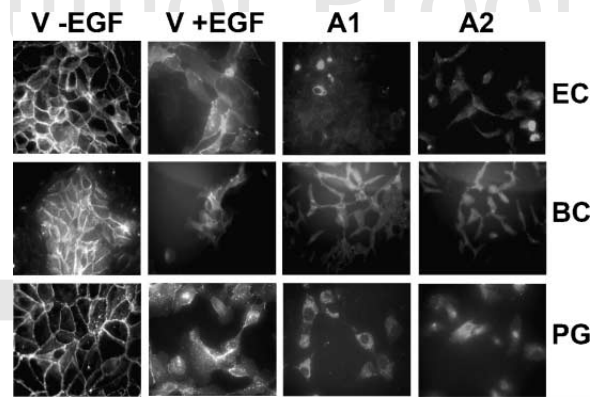


Figure 3. Detection of downregulation of junctional proteins in EGFRvIII-expressing cells by immunofluorescence microscopy. Immunofluorescence detection of E-cadherin (EC),  $\beta$ -catenin (BC), and  $\gamma$ -catenin/plakoglobin (PG) in vector control cells (V), and EGFRvIII-expressing clones A1 and A2. Where indicated, cells were treated with 25 nM EGF for 24 h. Vector control cells and parental OVCA 433 cell gave identical results staining for EC, and BC in the adherens junctions, and plakoglobin in the desmosomal junctions; whereas both vIII A1 and vIII A2 did not display functional junctions. This figure is representative of six independent experiments.

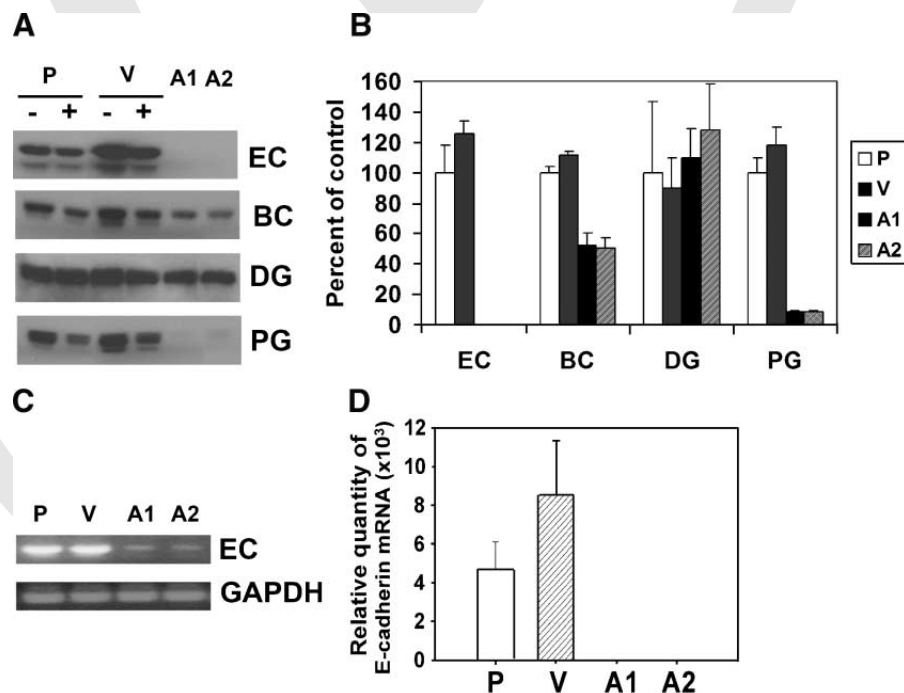


Figure 4. Downregulation of junctional proteins in EGFRvIII expressing cells. (A) Total cell lysate (10  $\mu$ g protein) was fractionated by PAGE. Junctional proteins were visualized by Western blot analysis with antibodies directed against EC, BC, desmoglein (DG), and PG. As indicated, (-) cells were treated with 25 nM EGF for 24 h. P, parental OVCA 433 cells; V, vector control cells; and A1 and A2 are EGFRvIII expressing clones. (B) Expression was quantitated by direct luminescence detection with a Kodak Image Station 440 CF. The results shown represent the values obtained for three independent isolates of the indicated cell lines  $\pm$  SD. Expression of EGFRvIII

resulted in significantly decreased EC ( $P=0.001$ ), PG ( $P=0.007$ ), and BC ( $P=0.020$ ), but desmoglein protein expression was not affected. (C) Reduced EC mRNA levels in EGFRvIII-expressing cells. EC mRNA was detected by RT-PCR in control (P=parental OVCA 433 cells; V=vector control cells) or EGFRvIII expressing cells (clones A1 and A2). GAPDH was amplified as an internal control. (D) mRNA of E-cadherin was quantitated by TaqMan real-time PCR as described under Materials and Methods, performed in triplicate  $\pm$  SD. The level of EC mRNA is 1500-fold lower in EGFRvIII-expressing cells in comparison to controls ( $P<0.001$ ).

(Figure 4A and B), which was consistent with immunofluorescence findings (Figure 3). This decrease in E-cadherin protein exceeded that observed with EGF treatment of parental or vector transfected OVCA 433 cells for 24 h (Figure 4A and B) or 5 d (data not shown). Extended EGF treatment (up to 10 d) of another epithelial ovarian cancer cell line, OVCA 429, also led to decreased E-cadherin expression to a level comparable to that detected in OVCA 433 (data not shown). E-cadherin mRNA was substantially decreased in EGFRvIII expressing cells when compared to parental or vector control cells (Figure 4C). Quantitation of E-cadherin mRNA in the different cell lines revealed that the level of E-cadherin mRNA was 1500-fold lower in EGFRvIII expressing cells than in control cells ( $P < 0.001$ ) (Figure 4D). These findings indicate that expression of the constitutively active EGFRvIII disrupts cell-cell junctions and downregulates a subset of junctional proteins. These changes likely contribute to the dissociated and migratory cell phenotype observed in EGFRvIII expressing cells.

#### EGFRvIII Expression Promotes Phenotypic Transition

It has been reported that N-cadherin expression, a marker of mesenchymal differentiation, increases with tumor grade in endometrioid and serous ovarian carcinomas [41]. We determined whether loss of E-cadherin in EGFRvIII-expressing cells was accompanied by an increase in N-cadherin and the acquisition of other indicators of a mesenchymal phenotype. The mesenchymal marker N-cadherin was elevated in EGFRvIII-expressing cells (Figure 5), suggesting a switch in cadherin-mediated cell adhesion. Furthermore, vimentin, an intermediate filament characteristic of mesenchymal cells, was elevated in EGFRvIII expressing cells (Figure 5). This was accompanied by decreased expression of the epithelial keratins (keratin 7, 8, and 18) and a mucin 1 fragment, another indicator of ovarian epithelial differentiation (Figure 5). Therefore both cadherins and intermediate filaments change from epithelial to mesenchymal forms as a consequence of EGFRvIII expression in ovarian carcinoma cells.

In order to determine whether the dissociated phenotype would be retained in vivo, vector control and EGFRvIII expressing cells were injected subcutaneously in the flank region of nude mice and the resultant tumors were analyzed for morphology and expression of cadherins and intermediate filament proteins (Figure 6). Control cells formed tubular structures (Figure 6 upper left panel) whereas EGFRvIII A1 cells were predominantly individualized cells that occasionally formed small clusters (Figure 6 lower left panel). Immunohistochemical staining revealed that most vector control cells were cytokeratin-positive and peripheral E-cadherin staining was evident (black arrows in the figure). In contrast, EGFRvIII A1 cells were cytokeratin-negative, a finding

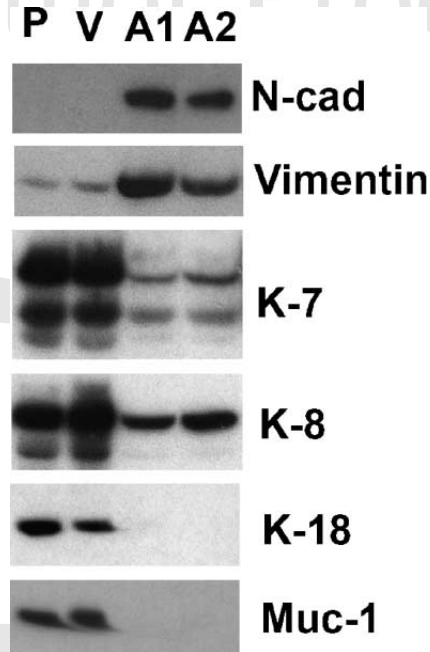


Figure 5. Western blot analysis of epithelial and mesenchymal markers. Total cell lysate (10  $\mu$ g protein) was fractionated by PAGE. The mesenchymal markers N-cadherin (N-cad), and vimentin or the epithelial markers keratin 7 (K-7), keratin 8 (K-8), keratin 18 (K-18), and mucin1 (MUC-1) were detected by Western blot analysis. B: Detection of vimentin in OVCA 429 cell line. P, parental OVCA 433 cells; V, vector-control cells; A1 and A2, EGFRvIII expressing clones A1 and A2, respectively. The results are representative of three independent experiments.

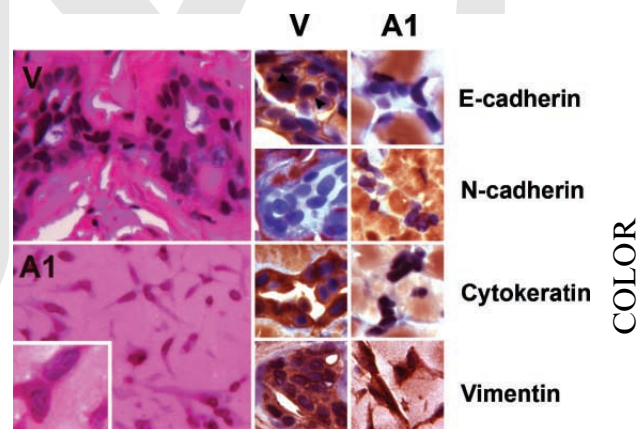


Figure 6. In vivo detection of epithelial and mesenchymal markers. In vivo, control cells formed tubular structures (upper left panel), whereas EGFRvIII A1 cells were mostly individualized cells (lower left panel) as detected by Hematoxylin & Eosin staining. The inset is a higher magnification of the H&E section of EGFRvIII A1. Cells in which cytoplasm and round to oval nuclei can be distinguished are tumor cells; stromal cells are mature fibroblasts with very small amounts of cytoplasm and condensed elongated nuclei. Immunohistochemical staining for EC (black arrows), N-cadherin, cytokeratins, and vimentin revealed loss of the epithelial markers (EC and cytokeratins), and increased expression of mesenchymal markers (N-cadherin and vimentin) in EGFRvIII-expressing cells in comparison to control cells.

consistent with their fibroblastic phenotype, and both EGFRvIII clones were E-cadherin-negative. Furthermore, tumors formed from the EGFRvIII expressing cells displayed elevated expression of N-cadherin and vimentin (Figure 6), in agreement with *in vitro* findings (Figure 5). These results indicate that the phenotypic and functional alterations conferred by EGFRvIII expression are retained in an *in vivo* environment and suggest that constitutive activation of the EGF receptor contributes to an epithelial to mesenchymal transition (EMT) in ovarian tumor cells.

### DISCUSSION

The constitutively active EGF receptor EGFRvIII is found at high frequency in many tumor types [26–28,30,33] and has been detected in primary ovarian tumors with expression reportedly more frequent in metastatic lesions when compared to primary tumors [26,42]. To date, the functional impact of EGFRvIII in ovarian cancer is unknown; however, several studies in other tumor types suggest a role for this mutant EGF receptor in tumor growth and metastasis. EGFRvIII expression is transforming in the absence of ligand, potentiates tumor xenograft growth in nude mice and promotes motility of fibroblasts, human colon cancer cell lines and neural stem cells [28,33,43–46]. Furthermore, recombinant single chain antibody-toxin directed against EGF receptor and EGFRvIII suppresses lung metastases in a model with EGFRvIII expressing murine renal cells [47]. These findings, in conjunction with clinical evidence for EGFRvIII expression in high-grade human tumors, suggest that EGFRvIII may contribute to a metastatic phenotype in a variety of cancers. In this study, we reported that expression of EGFRvIII in an epithelial ovarian tumor cell line promoted a motile, dispersed and fibroblastic phenotype with an associated gain of mesenchymal markers consistent with an EMT.

Many of the changes observed in EGFRvIII expressing cells are predicted to support a motile phenotype. Reduced expression of epithelial cytokeratins 7, 8, and 18 was accompanied by increased vimentin expression (Figures 5 and 6). Vimentin is associated with migratory behavior during developmental EMT and in cancer [48]. Experimental elevation of vimentin in a breast cancer model increases motility and invasiveness *in vitro* and conversely, downregulation of vimentin expression in highly invasive human breast cancer cells leads to decreased migration [49]. Furthermore, ascites-derived cells from NIH:OVCAR-3 ovarian tumor cells transplanted into athymic mice express higher levels of vimentin than their solid tumor counterparts [50]. Similarly, N-cadherin is thought to play a role in both stable and labile cellular interactions involved in migration [51]. Experimentally, N-cadherin induces a mesenchymal and scattered phenotype in squa-

mous cell carcinoma [52] and the extracellular domain of N-cadherin is sufficient to promote migration and EMT of squamous epithelial cells [53]. Elevated N-cadherin is linked to increased cell motility and to invasive and metastatic phenotypes in prostate and breast cancer cells [54–56]. Furthermore, N-cadherin expression in endometrioid and serous ovarian carcinomas increases with the tumor grade and reaches peak expression in metastases [41]. Taken together, these findings suggest gain of the mesenchymal markers N-cadherin and vimentin as a consequence of EGFRvIII expression may be associated with a more malignant phenotype in epithelial ovarian cancer.

We reported previously that EGF promotes colony dispersion and junctional disruption in several ovarian cancer cell lines [18,19]. Similarly, EGFRvIII expression disrupts adherens and desmosomal junctions (Figure 3) and this response is characterized by decreased E-cadherin,  $\beta$ -catenin, and plakoglobin protein levels (Figure 4). These changes in junctional components may be significant, as  $\beta$ -catenin expression is lost in 21% of ovarian carcinomas and reduced  $\beta$ -catenin is associated with a more motile and invasive phenotype [7]. Furthermore, reduced E-cadherin is characteristic of poorly differentiated invasive ovarian tumors [6,8] and E-cadherin expression is lower in ascites-derived cells and metastatic lesions of advanced ovarian cancer than the corresponding primary tumors [8,57]. Interestingly, transmission electron microscopy revealed disruption of adherens junctions when EGFRvIII was expressed in a small lung cancer cell line [35], indicating that modulation of junctional integrity by EGFRvIII is not restricted to ovarian tumor cells.

Recent discussions of EMT recognize that EMT encompasses a wide range of changes in epithelial plasticity [58]. More pronounced EMT that is characterized by disruption of adherens junctions, loss of E-cadherin, and gain of vimentin is associated with tumor metastasis [48,58,59], and all of these responses were observed following expression of the constitutively active EGFRvIII in an epithelial ovarian tumor cell line. A number of signaling events including, activation of receptor tyrosine kinases such as EGF receptor, can promote EMT in target cells [58,60]. Extended EGF stimulation of a breast cancer cell line leads to EMT as defined by upregulation of vimentin, downregulation of E-cadherin and elevated N-cadherin expression [61]. EGF can also induce EMT in normal OSE maintained in defined culture medium containing hydrocortisone [62] and EGF-dependent phenotypic plasticity of normal ovarian epithelium is observed in postovulatory repair processes [2,3]. Evidence for the importance of EGF family ligand autocrine loops in EMT has been reported [reviewed in 58] and our findings suggest that constitutive EGFR activity conferred by the EGFRvIII mutation may similarly lead to EMT in

appropriate target tissues. Based on this study, we propose that expression of EGFRvIII in epithelial ovarian cancer may contribute to metastatic dissemination through stimulation of an EMT.

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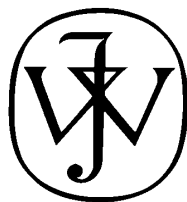
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Please add 5% Postage and Handling \$ \_\_\_\_\_

**TOTAL AMOUNT OF ORDER\*\*** \$ \_\_\_\_\_

*\*\*International orders must be paid in currency and drawn on a U.S. bank*

Please check one:  Check enclosed  Bill me  Credit Card

If credit card order, charge to:  American Express  Visa  MasterCard

Credit Card No \_\_\_\_\_ Signature \_\_\_\_\_ Exp. Date \_\_\_\_\_

**BILL TO:**

Name \_\_\_\_\_

Institution \_\_\_\_\_

Address \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**SHIP TO:** (Please, no P.O. Box numbers)

Name \_\_\_\_\_

Institution \_\_\_\_\_

Address \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**Purchase Order No.** \_\_\_\_\_

Phone \_\_\_\_\_ Fax \_\_\_\_\_

E-mail \_\_\_\_\_

## **Softproofing for advanced Adobe Acrobat Users - NOTES tool**

NOTE: ADOBE READER FROM THE INTERNET DOES NOT CONTAIN THE NOTES TOOL USED IN THIS PROCEDURE.

Acrobat annotation tools can be very useful for indicating changes to the PDF proof of your article. By using Acrobat annotation tools, a full digital pathway can be maintained for your page proofs.

The NOTES annotation tool can be used with either Adobe Acrobat 4.0, 5.0 or 6.0. Other annotation tools are also available in Acrobat 4.0, but this instruction sheet will concentrate on how to use the NOTES tool. Acrobat Reader, the free Internet download software from Adobe, DOES NOT contain the NOTES tool. In order to softproof using the NOTES tool you must have the full software suite Adobe Acrobat 4.0, 5.0 or 6.0 installed on your computer.

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