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Identification of epidermal growth factor-responsive genes in normal rat ovarian surface epithelial cells

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Abstract

Alteration in epidermal growth factor receptor (EGFR) family signaling is among the most frequently implicated effectors of human oncogenesis. Overexpression of members of this family of receptors has often been detected in many epithelial tumors and is believed to be associated with an overall poor prognosis in patients with cancer. Therefore, we hypothesized that identification of potential EGF target genes in normal cells will provide a basis for unbiased genetic analysis of this signaling pathway in cancer. We utilized Atlas Rat 1.2 nylon cDNA arrays (Clontech) to determine gene expression changes in normal rat ovarian surface epithelial (ROSE) cells following EGF treatment. The results indicate activation of genes involved in a wide variety of cellular mechanisms, including regulation of cell cycle and proliferation, apoptosis, and protein turnover. In addition, using an in vitro model of ovarian cancer, we demonstrated that malignant transformation of ROSE cells resulted in alteration of downstream effectors of the EGFR pathway, as exemplified by aberrant expression of *p66Shc*, *c-Jun*, *c-Myc*, c-*Fos*, *Lot1*, *p21Cip/Waf*, and *cdc25A*. These data suggest that knowledge of the downstream genetic lesions, which may result in loss of growth factor requirement of the affected cells, will be crucial for the selection of the EGFR pathway as an effective target for cancer therapy.

Keywords: EGF; Ovarian cancer; Ovarian surface epithelia; Microarray

The EGF/ErbB family of receptor tyrosine kinases plays a key role in normal development and cell growth regulation of the epithelial tissues, including ovarian surface epithelia. In many instances, cancer cells arise from the normal epithelial tissues as a result of genetic aberrations in growth factor signaling pathways [1]. Alterations in the EGF family signaling pathways have been found to be frequent genetic lesions implicated in human cancer [2,3]. Ligand (EGF or TGF-α) binding to the 170-kDa transmembrane glycoprotein receptor triggers the activation of signaling cascades that connect the activated receptor at the cell surface with the nucleus, including signals transduced by the Erk kinases, phosphoinositide 3′-kinase (PI3K), and members of the STAT family of transcription factors [4–7]. Expression of EGF and TGF-α has been documented in a variety of tumor cells, including ovarian cancers [8–10], and their role in stimulating growth has been reported in ovarian cancer cell lines [11–13].

Several genetic events are known to alter the EGFR function. For example, a truncated form of EGFR has been observed in tumors, which can adversely affect prognosis [14–17]. The EGFR gene is often amplified, overexpressed, or mutated in many types of tumors, including carcinomas of the ovary [12,17–21], breast [22], and bladder [23], and in glioblastomas [24–26]. The type III EGF receptor (EGFRvIII) is the result of a large, spontaneously occurring in-frame deletion of the extracellular ligand-binding domain of the receptor. This type of alteration was found in a high percentage of primary human brain, breast, lung, and ovarian tumors [27]. It appears that cell transformation associated with EGFRvIII mutant results from the constitutive activation of the receptor, which in turn leads to the constitutive activation of PI3 kinase.

Ligand-independent signaling by the EGF receptor has been shown to result in qualitative changes in

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downstream targets of the receptor, which specifically result in oncogenic signaling, transformation, and tumorigenicity [28]. Together, these studies suggest that deregulation of EGFR/ErbB receptor family members contributes to the etiology and progression of epithelial ovarian cancer. Several approaches, including targeting EGFR with antagonistic antibodies or small-molecule tyrosine kinase inhibitors, have been developed [29] as a means of controlling cancer. While these tools have therapeutic values, they may be ineffective in some patients due to acquired resistance. Therefore, a better understanding of the cellular and molecular mechanisms of the EGF receptor signaling pathway will be useful in explaining the de novo acquired resistance to the inhibitors. Particularly, identification of downstream effectors of EGF in normal cells will provide a basis for the clinical analysis and management of specific genetic lesion(s) of the EGFR signaling pathway in cancer. While it is known that EGF is produced in the ovary and influences proliferation of the malignant ovarian surface epithelium (OSE), its role in malignancy or in regulating the normal OSE is unclear [30]. Recently, we have presented evidence suggesting that in normal OSE cells the zinc-finger transcription factor LOT1 can transduce signals originating from EGFR [31]. In this study, we have carried out a broader examination of gene expression changes in the normal and malignantly transformed ROSE cells, in the presence or absence of EGF stimulation, to gain a better understanding of the components of this important signaling pathway.

Materials and methods

Cells and cell cultures. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Gaithersburg, MD) supplemented with 4% fetal bovine serum, glutamine (2 mM), insulin (10 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37 °C. For growth factor stimulation, cells were treated 5–7 days after plating, when they were almost confluent and quiescent [32]. Normal rat ovarian surface epithelial (ROSE) cells were obtained from the ovaries of adult Fisher rats by selective trypsinization [33,34]. Development of the in vivo model of rat OSE cancer and derivation of the tumor ROSE cell line NuTu 26 have been described previously [32–34].

Analysis of cDNA microarray. Rat Atlas 1.2 Arrays (Clontech, Palo Alto, CA) were used for comparing the gene expression profiles between the controls and EGF-treated normal ROSE cells. Total RNA was purified from treated and untreated cells using an acid phenol/chloroform extraction procedure, according to the manufacturer's instructions (Atlas Pure Total RNA Labeling System; Clontech). The RNA was treated with 10 U DNase I for 30 min at 37 °C to remove any traces of genomic DNA contamination. The purity of the RNA samples was verified by PCR amplification of a GADPH gene intron–exon segment. To synthesize ^{33}P-labeled probes, 1–3 μg of total RNA was combined with the mixture of Atlas Rat 1.2 gene-specific oligonucleotide primers (Clontech, Palo Alto, CA). The solution was preheated to 94 °C and annealed at 70 °C for 10 min followed by extension at 49 °C for 35 min in a 50 mM Tris–HCl (pH 8.3) buffer, containing 75 mM KCl, 3 mM $MgCl_2$, 2.5 mM DTT, 0.5 mM dNTPs, and 35 μCi [^{33}P]dCTP (3000 Ci/mmol; NEN, Boston, MA), and 200 U of Superscript II (Life Technologies, Gaithersburg, MD). The reaction was stopped at 94 °C for 5 min and then placed in an ice bath. Unincorporated [^{33}P]dCTP was removed on a Microspin Sepharose G-50 gel filtration column (Amersham–Pharmacia Biotech, Piscataway, NJ) and the probe was hybridized to the Clontech Rat Atlas 1.2 membranes in a hybridization buffer provided by the array manufacturer (Clontech) for 18–24 h at 64 °C. Non-hybridized probe was washed according to the manufacturer's instructions and the arrays were subjected to multiple autoradiographic exposures of variable length on Kodak Biomax film using LE intensifying screens. The obtained autoradiographic images were scanned at 16 bit/1200 dpi (20 μm) resolution and pre-processed to 8 bit/200 dpi bitmap files in Adobe PhotoShop. The array images were then processed using *ArrayExplorer* software [35] to analyze gene expression levels. Hybridization signals from housekeeping genes were used to normalize the samples by linear regression.

Northern and Western blots. Total RNA used for array gene expression analysis, or alternatively isolated from cells by the guanidinium isothiocyanate extraction method [36] was separated on 1% agarose gels containing 2.2 M formaldehyde and subjected to Northern blot analysis. The RNA was transferred to Nylon membranes (Micron Separations) by capillary action and hybridized as described previously [32,37]. Western blot analysis was performed with whole cell lysates prepared with 250 μl/well M-PER Protein Extraction Reagent (PIERCE, Rockford, IL), supplemented with 2 mM PMSF and 0.5 mM DTT. Total proteins (15 μg/lane) from each sample were resolved by SDS–PAGE (10%) followed by transfer to a Hybond nylon membrane (Amersham Life Science, UK) for Western blot analysis. Immunoblots were blocked overnight in 5% nonfat dried milk (w/v) in TBST containing 20 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20. Blots were incubated for 1 h with corresponding monoclonal antibodies diluted (1:1000 or as recommended by the manufacturers) with 1% nonfat dried milk in TBST. The blots were washed with TBST three times and incubated for 1 h with a secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (Amersham–Pharmacia Biotech, UK). The protein bands were detected by the Western Lightning Chemiluminescence Reagent (Perkin–Elmer Life Sciences, Boston, MA) and exposure to X-ray films (Kodak, Rochester, NY). The antibodies were obtained from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA).

Results and discussion

Identification of EGF-responsive genes in normal ROSE cells

As was noted above, the EGF-responsive pathway plays a crucial role in growth and proliferation of many types of cells, including ovarian epithelial cells. In this study, we reasoned that identification of different genes in this pathway would provide a basis for future clinical and therapeutic applications. Towards understanding the molecular mechanisms by which EGFR transmits mitogenic messages to the downstream signaling pathways in OSE cells, we used normal cells to avoid genetic disruption or complexity, which might be associated with the transformed cells. We have utilized both traditional and microarray procedures to analyze the gene expression profile of untreated (control) and EGF-treated normal ROSE cells. The normal ROSE cells

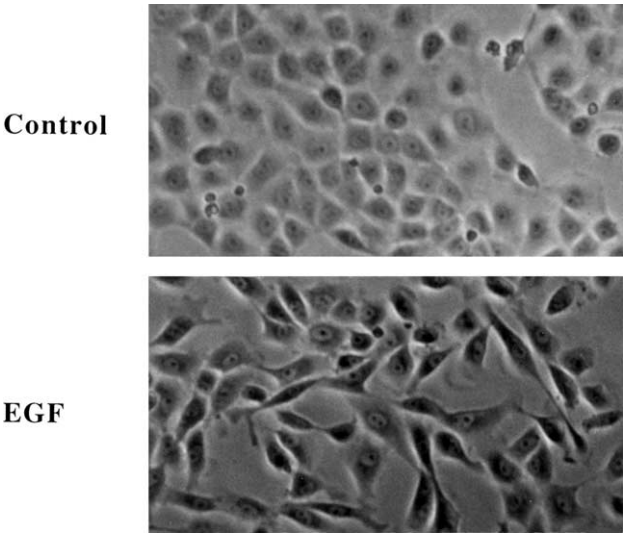

Fig. 1. Morphological changes of normal ROSE cells treated with EGF. The cells were grown in DMEM (5% fetal bovine serum) in the absence (control) or presence of EGF and used for phase contrast microscopy.

were grown to near confluence or quiescence and treated with or without EGF (250 ng/mL) for 5 h. Effectiveness of the treatment was judged by the morphological changes in the normal ROSE cells from cobblestone-like to fibroblastic (elongated) shape (Fig. 1). Total RNAs purified from corresponding cell cultures were then used to hybridize Rat Atlas 1.2 nylon cDNA arrays (Clontech) containing 1200 genes, or for Northern blot analysis. Microarray data analysis identified about 100 genes, whose expression was upregulated in response to EGF treatment. The results are presented in Table 1. A large number of upregulated genes are known to be involved in a wide variety of cellular processes, including cell cycle regulation, growth, apoptosis, and protein turnover. A summary list of these genes is presented in Table 2. Among the genes being upregulated in normal ROSE cells are *plasminogen activator-1* (*PAI-1*), *ornithin decarboxylase*, *urokinase receptor*, *Egr1*, *TIMP-1*, *protein phosphatase 2C*, *14-3-3* genes, *RIN1*, *proteasome* genes, *AKT1* and *2*, *calmodulin*, *MAPKK2*, *hnRNP K*, *cyclin D1* and *D3*, *GST P* subunit, *Max*, *c-Jun*, *ARF1*, and *ARF6*. Some of these genes have previously been demonstrated to be upregulated by EGF and their products may be linked to cancer development or progression.

In this study, we found that *PAI-1* and *TIMP-1* (tissue inhibitor of matrix metalloproteinase) are upregulated by EGF in normal ROSE cells (Tables 1 and 2). Similarly, induction of *PAI-1* and *TIMP-1* by EGF has previously been observed in several ovarian cancer cell lines and it has been reported that proteinase activity is required for EGF-induced ovarian cancer cell invasion [38]. *PAI-1* was found to be a potent regulator of angiogenesis and its elevated expression is associated with poor prognosis in many cancers [39]. The transcription factor *Egr1* was among the genes found to be strongly upregulated by EGF in normal ROSE cells in this study, as well as in other studies using different cell lines such as MDA-MB-361 breast cancer cell line [40]. Interestingly, *Egr1* can induce *PAI-1* expression [40]. *Ornithine decarboxylase* (*ODC*), another gene upregulated in the normal ROSE cells, is a rate-limiting enzyme in polyamine biosynthesis and is often associated with initiation of normal cell growth and with sustained neoplastic cell growth. ODC-induced transformation of NIH-3T3 cells is mediated, at least in part, by alterations in EGFR signal transduction activity [41]. Recently, it was found that transformation of cells by *H-ras* results in aberrant regulation of the *ODC* gene [40].

Another gene also found upregulated in EGF-treated normal ROSE cells is *hnRNP K* (Tables 1 and 2). This latter gene has been shown to be induced in breast cancer cells by EGF and its overexpression significantly increased *c-Myc* promoter activity and enhanced breast cancer cell proliferation and growth in an anchorage-independent manner [42]. hnRNP K protein has been implicated in such functions as sequence-specific DNA binding and transcription, RNA binding, and nucleocytoplasmic shuttling [42], and it is a component of an intronic splicing enhancer [43]. The 14-3-3 proteins comprise a family of 30 kDa acidic proteins, which interact with a wide variety of cellular proteins. For example, the activity of the proapoptotic ligand BAD in the ovary is regulated by upstream follicle survival factors through its binding to 14-3-3 protein [44]. RIN1 is a component of the EGF pathway and was originally identified by its ability to bind and inhibit activated Ras, and by its interaction with c-ABL and 14-3-3 proteins [45]. ADP-ribosylation factors (ARFs) are small 20-kDa guanine nucleotide-binding proteins and are members of the Ras superfamily [46]. They are also associated with the EGF pathway and play critical roles in a number of different vesicle-trafficking pathways in eukaryotic cells. Therefore, it is relevant that in this study the genes *ARF1* and *ARF6* were among the genes induced by EGF stimulation of the ovarian epithelial cells.

Signals initiated by growth factors trigger quiescent cells to advance into the first gap phase (G_1) of the cell division, traverse the G_1 phase and then become committed to DNA synthesis or S phase [3,47]. The cells require sustained exposure to EGF for at least 6–8 h before they are committed to DNA synthesis and transition through the G_1 phase [14,48–51]. In the present report, we have identified two cell cycle regulators, Cyclin D1 and Cyclin D3, whose expression is markedly induced in normal ROSE cells following EGF treatment. Similar results of significant Cyclin D1 and D3 induction were obtained in primary cultures of normal rat hepatocytes stimulated with EGF [52]. The data from this study indicate that the expression of these two

Table 1
Identification of the EGF-responsive genes in normal ROSE cells by Atlas 1.2 microarray analysis

Gene description	GenBank #	Fold
bcl-2-Associated death promoter (BAD)	AF003523	2.0
Proteasome β subunit precursor; macropain β; multicatalytic endopeptidase complex β	L17127	2.1
Cofilin	X62908	2.1
c-jun Proto-oncogene; transcription factor AP-1; RJG-9	X17163	2.1
Glutathione *S*-transferase P subunit; GST subunit 7 π (GST7-7)	X02904	2.1
Acyl-CoA oxidase	J02752	2.1
Aminopeptidase B	U61696	2.1
Matrix metalloproteinase 14 precursor (MMP14); membrane-type matrix metalloproteinase 1	X83537	2.1
G1/S-specific cyclin D3 (CCND3)	D16309	2.2
cAMP-dependent protein kinase type I-α regulatory chain	M17086	2.2
G_1/*S*-specific cyclin D1 (CCND1)	D14014	2.2
Proteasome ι subunit; macropain ι subunit; multicatalytic endopeptidase complex ι subunit	D10755	2.2
Orphan nuclear receptor TR4; NR2C2	L27513	2.2
Inositol triphosphate receptor, type 2 (ITPR2)	X61677	2.3
dC-stretch binding protein (CSBP); heterogeneous nuclear ribonucleoprotein K (HNRNP K)	D17711	2.3
60S ribosomal protein L44; L36A	M19635	2.3
Na^+/K^+ ATPase α1 subunit	M28647	2.4
Cyclin-dependent kinase 4 (CDK4); cell division protein kinase 4; PSK-J3	L11007	2.4
NDK-B; nucleoside diphosphate kinase B; metastasis-reducing protein; c-myc-related transcription factor	M91597	2.5
Casein kinase I δ; CKId; 49-kDa isoform	L07578	2.5
Epidermal fatty acid-binding protein (E-FABP); cutaneous fatty acid-binding protein (C-FABP)	U13253	2.5
Potassium channel Kir6.2, inward rectifier, ATP-sensitive	X97041	2.5
ATPase, subunit F, vacuolar (vatf)	U43175	2.5
Tissue carboxypeptidase inhibitor (TCI)	U40260	2.6
Sodium/hydrogen exchange protein 1	M85299	2.6
BAX-α	U49729	2.6
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	M17701	2.6
GTP-binding protein G(i)/G(s)/G(o) γ-9 subunit; γ8	L35921	2.6
ATP synthase lipid-binding protein P1 precursor; ATPase protein 9; and ATP5G1	D13123	2.7
SHPS-1 receptor-like protein with SH2 binding site	D85183	2.7
Neuron-specific enolase (NSE); γ enolase (EC 4.2.1.11); 2-phospho-D-glycerate hydrolase	AF019973	2.7
Mitochondrial ATP synthase D subunit; ATP5H	D10021	2.7
67-kDa glutamic acid decarboxylase (GAD67); GAD1	M34445	2.7
HSP84; HSP90-β; heat shock 90 kDa protein	S45392	2.8
Ezrin; cytovillin; villin 2 (VIL2); p81	X67788	2.8
Presenilin 1 (PSNL1; PSEN1; PS1); S182 protein	D82363	2.8
Calcium/calmodulin-dependent protein kinase type IV (CAMK IV; catalytic chain); CAM kinase	M63334	2.8
Mitogen-activated protein kinase p38 (MAP kinase p38); CSBP2	U73142	2.8
SR13 myelin protein; peripheral myelin protein 22 (PMP-22); CD25 protein	M69139	2.8
rac-β serine/threonine kinase (rac-PK-β); AKT2	D30041	2.9
Tumor necrosis factor receptor 1 precursor (TNFR1)	M63122	2.9
Mitochondrial ATP synthase β subunit precursor (ATP5B)	M19044	2.9
Somatostatin receptor 5 (SSTR5; SS5R)	L04535	2.9
Calmodulin (CALM; CAM)	X13817	2.9
Vacuolar ATP synthase 16-kDa proteolipid subunit; ATP6C; MVP; ATPL	D10874	3.0
Proteasome subunit RC6-1	D30804	3.0
Macrophage migration inhibitory factor (MIF)	U62326	3.0
G protein coupled receptor 1	S74702	3.0
Extracellular signal-regulated kinase 2 (ERK2); mitogen-activated protein kinase 2 (MAP kinase 2)	M64300	3.1
ADP-ribosylation factor 3 (ARF3)	L12382	3.2
Lysosphingolipid, G protein-coupled receptor	U10699	3.2
Dual-specificity mitogen-activated protein kinase kinase 2 (MAP kinase kinase 2); MAPKK2	D14592	3.3
ras-related protein m-ras	D89863	3.3
rac-α serine/threonine kinase (RAC-PK-α); protein kinase B (PKB); AKT1	D30040	3.3
Hypoxanthine–guanine phosphoribosyltransferase (HPRT)	M63983	3.3
ATPase, transitional endoplasmic reticulum	U11760	3.4
Cytochrome *c* oxidase, subunit Va, mitochondrial	X15030	3.4
Protein tyrosine phosphatase PTPase	M33962	3.5
Proteasome δ subunit precursor; macropain δ; multicatalytic endopeptidase complex δ	D10754	3.6
Interleukin-4 receptor	X69903	3.6
Triacylglycerol lipase precursor (hepatic)	M16235	3.7
14-3-3 protein ζ/δ; PKC inhibitor protein-1; KCIP-1; mitochondrial import stimulation factor	D17615	3.7
Max; c-myc dimerization partner and coactivator	D14447	3.7

Table 1 (*continued*)

Gene description	GenBank #	Fold
Fructose–bisphosphate aldolase A (ALDOA); muscle-type aldolase	M12919	3.8
Proteasome subunit C5	X52783	3.9
ATPase, proteasomal, liver, TBP1	U77918	3.9
Casein kinase II β subunit (CKII; CSNK2B; CK2N); phosvitin	L15619	3.9
LIM domain kinase 2 (LIMK2)	D31874	4.0
D(4) dopamine receptor; D(2C) dopamine receptor	M84009	4.1
Lipocortin 2	S73557	4.1
Protein arginine *N*-methyltransferase 1	U60882	4.1
Tubulin α-1 (TUBA1)	V01227	4.2
Transforming growth factor β (TGF-β) masking protein large subunit	M55431	4.2
Structure-specific recognition protein 1 (SSRP1); recombination signal sequence recognition protein	L08814	4.3
Vasopressin V2 receptor	Z11932	4.3
SURVIVAL OF MOTOR NEURON(RSMN)	U75369	4.4
Proteasome component C3	J02897	4.4
ADP-ribosylation factor 1 (ARF1)	L12380	4.4
Antigen peptide transporter 1	X57523	4.5
Interleukin-1 receptor type I (IL-1R-1); P80	M95578	4.6
14-3-3 protein epsilon; PKC inhibitor protein-1; KCIP-1; mitochondrial import stimulation factor	M84416	4.7
c-H-ras proto-oncogene; transforming G-protein p21	M13011	4.7
Annexin V (ANX5); lipocortin 5; placental anticoagulant protein I (PAP-I); endonexin II; calphobidin I	M21730	4.8
Low-density lipoprotein receptor precursor (LDL receptor; LDLR)	X13722	4.8
G_1/S-specific cyclin D2 (CCND2); vin-1 proto-oncogene	D16308	4.8
glutathione synthetase (GSH synthetase; GSH-S; GSS); glutathione synthase	L38615	4.9
ADP-ribosylation factor 6 (ARF6)	L12385	5.0
Calponin, acidic	U06755	5.1
Nm23-M2; nucleoside diphosphate kinase B; metastasis-reducing protein; c-myc-related transcription factor	D13374	5.3
Prohibitin (PHB); B-cell receptor-associated protein 32 (BAP32)	M61219	5.3
Cyclin-dependent kinase 5 (CDK5); τ protein kinase II (TPKII) catalytic subunit; PSSALRE kinase	L02121	5.3
CD3, γ chain	S79711	5.5
Protein phosphatase 2C isoform; Mg^{2+}-dependent protein phosphatase β isoform	S90449	5.7
NADPH–cytochrome P450 reductase (CPR); POR	M12516	6.8
RIN1; interacts directly with Ras and competes with Raf1	U80076	9.1
Transforming growth factor, β1	X52498	9.8
Ornithine decarboxylase (ODC)	J04791	10.0
Fibroblast ADP/ATP carrier protein; ADP/ATP translocase 2; adenine nucleotide translocator 2	D12771	11.0
Tissue inhibitor of metalloproteinase-1 (TIMP-1)	L31883	11.0
Early growth response protein 1 (EGR1); nerve growth factor-induced protein A (NGFI-A)	M18416	11.1
Urokinase receptor + GPI-anchored form urokinase plasminogen activator surface receptor	AF007789	18.5
Plasminogen activator inhibitor-1 (PAI-1)	M24067	34.5

The table shows the genes found to be upregulated by EGF treatment of the cells (see Materials and methods).

cell cycle regulators may also be important in the G_0/G_1 transition and cell cycle progression of primary cultures of normal OSE cells. Induction of Cyclin D1, as well as B/Akt activation, by EGF has also been reported elsewhere [53]. Taken together, we believe that the results presented here could be very useful in assessing the role of the EGFR signaling pathway both in normal and in malignant cells, and particularly in ovarian epithelial cancer cells for which no data exist on the role of many of these genes identified in this study (Tables 1 and 2).

Altered EGFR pathway in the rat model of ovarian cancer

Previously, we used an in vitro model of epithelial ovarian cancer to identify differentially expressed genes in comparison with the normal progenitor cells [32]. As a result, we cloned the novel gene *Lot1*, based on its decreased expression in the malignant cells. We have also shown that *Lot1* expression is downregulated following activation of the EGFR signaling pathway in normal ROSE cells [31]. Based on these results, we reasoned that malignant transformation of the cells might be associated with aberrant regulation of genes functioning downstream of EGFR. To test this hypothesis, we used the NuTu 26 cell line, in an in vitro model system of ROSE cells with a growth factor-independent and aggressive proliferative phenotype (Fig. 2; and data not shown) [31]. The results reported here demonstrate that the NuTu 26 cells harbor major genetic lesions in the EGFR pathway. In normal ROSE cells, EGF treatment induces the expression of a number of genes, including *c-Fos*, *c-Jun*, *Cip1/Waf1*, *c-Myc*, *cdc25A*, and *Egr1*, although at different time points (Fig. 3). In contrast, treatment of these cells with EGF downregulates the *Lot1* gene and the effect is persistent in the presence of the growth factor. In the malignant

Table 2
Representation of the selective genes upregulated by EGF in normal ROSE cells

Genes
PAI-1
EGR1
TIMP-1
ODC
RIN1
CD3 γ chain
CDK5
Prohibin
Nm23-M2
Calponin, acidic
ARF1 and ARF6
Glutathione synthase
Protein phosphatase 2C
Proteasome, C3; C5; δ; RC6-1; β subunit precursor
14-3-3 protein ϵ; ζ/δ; ι subunit
Calmodulin
Annexin V
Tubulin α-1
LIM domain kinase
Max
Interleukin 4 receptor
AKT1; 2
CAMKIV
hnRNP K
Cytochrome *c* oxidase
MAPKK2
Lysophospholipid GPCR
Presenilin 1
Ezrin
CDK4
Inositol triphosphate receptor, type 2
HSP84SHPS-1 receptor-like protein
Protein tyrosine phosphatase
TNFR1 precursor
G1/S-specific cyclin D1; D3
TR4
Bax-α
MMP14
BAD
TGF-β1
Glutathione *S*-transferase
IL-1R-1
Protein arginine *N*-methyltransferase
c-Jun protooncogene
TGF-β masking protein large subunit
Urokinase receptor

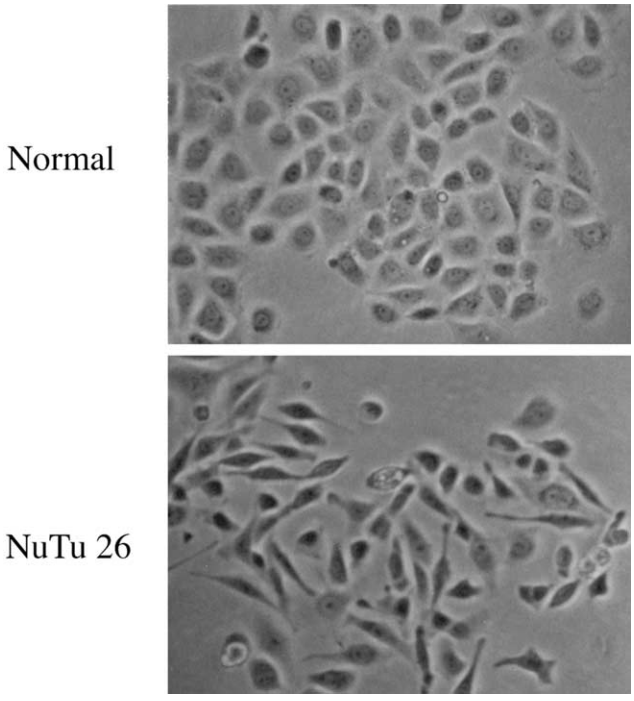

Fig. 2. Morphological characteristics of the tumor cell line NuTu 26 and normal ROSE cells. The cells were grown in DMEM (5% fetal bovine serum) and used for phase contrast microscopy.

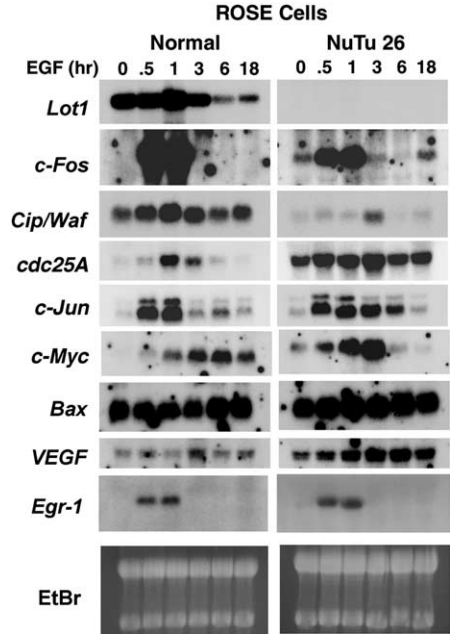

Fig. 3. Northern blot analysis of selected EGF-responsive genes in normal ROSE cells and NuTu 26 tumor cell line following growth factor stimulation. Total RNA (25 μg/lane) was hybridized to different ^{32}P-labeled probes. The figure shows alteration of the EGFR pathway in the rat model of ovarian cancer.

NuTu 26 cells, however, the patterns of gene expression are somewhat different (Fig. 3). The results from Northern blot analysis presented in Fig. 3 indicate that the normal expression pattern of a number of representative genes is deregulated in NuTu 26 cells. For example, the expression of *Lot1*, while present in the normal cells, is constitutively lost in NuTu 26 cells (Fig. 3). Similarly, *p21Cip1/Waf1* gene expression is found to be upregulated within 30–60 min following EGF stimulation, whereas in NuTu 26 cells the expression of the gene is significantly lower. In contrast, the expression of *cdc25A* is higher in the NuTu 26 cells as compared to the normal ROSE cells, both at the mRNA (Fig. 3) and protein (Fig. 4) levels. It has been reported

previously that this cell cycle phosphatase is a transcriptional target of c-Myc [54]. Interestingly, in this study, although *c-Myc* induction by EGF occurred in both the normal and tumor cells, the effect was more persistent in the normal cells (Fig. 3).

The AP1 family of transcription factors is composed of Jun family members, which form homo- or heterodimers among themselves as well as heterodimers with Fos family members. We examined the expression of two of these genes, *c-fos* and *c-Jun*, in normal ROSE cells and NuTu 26 cancer cells. As expected, both of these genes were induced rapidly but transiently following EGF treatment of the cells (Fig. 3). However, *c-Fos* appeared to be rapidly induced (within 30 min) and to a higher level in the normal cells than in the tumor cells. In contrast, the early response gene *c-Jun* was induced within 30 min upon EGF treatment of both cell types; however, its induction in the normal ROSE cells was transient and diminished to background levels 1 h after treatment, while its expression was sustained up to 6 h in NuTu 26 cells. The data in Fig. 3 also show that the level of VEGF expression, although not induced by EGF, is lower in the normal ROSE cells than in the tumor cell line.

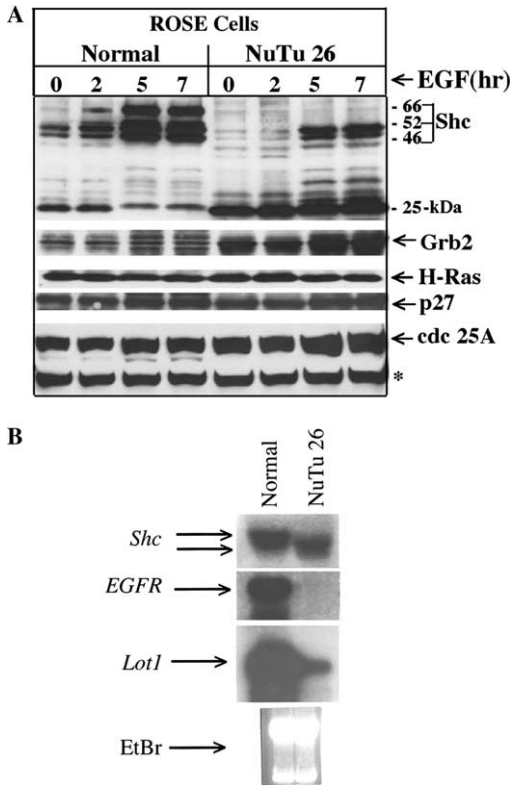

Fig. 4. (A) Western blot analysis of selected proteins in the EGF receptor pathway. Total protein was extracted from normal ROSE cells and NuTu 26 tumor cells stimulated by EGF (250 ng/ml) and used (15 μg/lane) for the Western blot analysis. The asterisk indicates a nonspecific protein signal as shown for loading comparisons. (B) Northern blot analysis of *Shc*, *Lot1*, and *EGFR* genes in normal ROSE cells and NuTu 26 tumor cell line. Loss or reduced expression of these genes in NuTu 26 tumor cell line is shown in Fig. 3B.

Expression of the adaptor protein p66Shc is lost in the tumor cell line NuTu 26

We also examined the expression of the adaptor protein Shc using Western blot analysis. The results are shown in Fig. 4A. We found that expression of this protein, which was found to be generally higher in the normal cells, is increased upon EGF treatment in both the normal and NuTu 26 cells. Strikingly, the data presented in Fig. 4 indicate that malignant transformation of rat OSE cells results in loss of the p66Shc isomer. The Shc protein family includes also p52 and p46 isomers [55], which together with p66Shc represent important proximal targets of the EGF receptor. The loss of p66Shc in the tumor NuTu 26 cell line appears to be relevant for the malignant phenotype of the cells, since expression of this adaptor protein has been previously shown to markedly accelerate the inactivation of the ERK pathway. It may therefore function in a dominant-interfering manner by inhibiting EGF receptor downstream signaling pathways [56]. It is believed that the serine/threonine phosphorylation of p66Shc impairs its ability to associate with the tyrosine-phosphorylated EGF receptor. Downregulation of p66Shc is also evidenced by the Northern blot analysis data (Fig. 4B), which show the absence or a decreased expression of this isomer. These data suggest that the p66Shc adaptor protein may play an important role in the development and/or progression of the OSE cancers. We also examined the changes in expression of Grb2 and H-Ras following treatment of the same cells with EGF. As shown in Fig. 4, the expression of these two genes remained unchanged, although the level of Grb2 is overall higher in the tumor cells, as compared to the normal progenitor ROSE cells.

We were also interested in comparing the expression of EGFR in the normal ROSE cells and the tumor cell line NuTu 26. Surprisingly, we found that the expression of EGFR mRNA is lower in the NuTu 26 cells as compared to the normal progenitor cells (Fig. 4). Similar results were obtained by using different domains of the receptor (extracellular, transmembrane, and cytoplasmic) as probes for the Northern blot analysis.

Taken together, the data shown in this report clearly show that, while some of the upstream elements of the EGFR pathway appear to be intact, the expression and regulation of some downstream effectors are altered. It is possible that the observed low expression of the receptor mRNA may be due to a negative feedback regulation. Therefore, utilization of EGFR expression data as a prognostic and/or therapeutic marker may not be an effective approach and should be paralleled with genetic data from downstream elements of the EGFR pathway.

These data indicate that the use of antagonistic anti-EGFR antibodies or inhibitory drugs would not be useful for cancer therapy when the pathway is constitutively operative independently of the receptor's activity.

Alteration of the EGFR pathway in the human ovarian cancer cells

As was noted above, in this study we found that the signaling pathway originating from EGFR is genetically altered in the tumor cell line NuTu 26. Therefore, we were interested to see whether a similar situation exists in human ovarian cancer. A number of human ovarian cancer cell lines were analyzed to determine the expression of the *LOT1* gene, as a paradigm, in comparison with *EGFR* expression. It appears that, while in some of the examined cancer cell lines the expression of *LOT1* and *EGFR* is responsive to EGF stimulation, in others it remains unchanged (Fig. 5). From the same data in Fig. 5 it is evident that the expression of these two genes is not proportional in the different cell lines. For example, in the ovarian cancer cell line PEO1, which expresses a high level of *EGFR*, expression of *LOT1* is either lost or minimal. In contrast, the expression of both genes is relatively high in OVCAR4, but very low in OVCAR10. Again, these results demonstrate that the identification of downstream genetic lesions along the EGF signaling pathway is important for the prognosis and management of these cancers. In conclusion, the data presented in this study are significant for understanding the normal physiology of the OSE cells with regard to the regulation of the EGFR pathway, and may facilitate the interpretation of the responses of the ells to EGF treatment, as well as the importance of this pathway in OSE cancer development and/or progression.

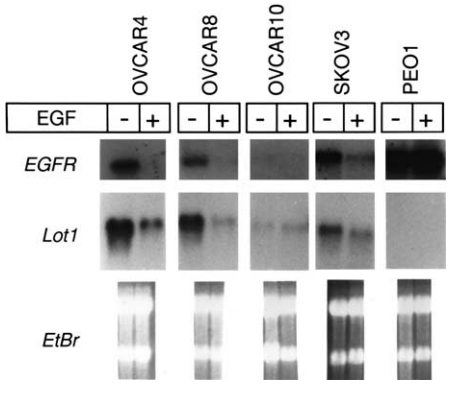

Fig. 5. Altered EGFR pathway in the human ovarian cancer cells. The human ovarian cancer cell lines were grown in RPMI (10% fetal bovine serum) to near confluence and treated with EGF (250 ng/ml) for 5 h. Total RNA (25 μg/lane) was hybridized with the ^{32}P-labeled probes.

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