The Granulin-Epithelin Precursor/PC-Cell-derived Growth Factor Is a Growth Factor for Epithelial Ovarian Cancer

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Abstract

Purpose: The role of growth factors in ovarian cancer development and progression is complex and multifactorial. We hypothesized that new growth factors may be identified through the molecular analysis of ovarian tumors as they exist in their native environment.

Experimental Design: RNA extracted from microdissected serous low malignant potential (LMP) and invasive ovarian tumors was used to construct cDNA libraries. A total of 7300 transcripts were randomly chosen for sequencing, and those transcripts were statistically evaluated. Reverse transcription-PCR and immunohistochemistry were used to validate the findings in tumor tissue samples. Ovarian cancer cell lines were used to test gene effects on monolayer growth, proliferative capacity, and density-independent growth.

Results: Analysis of the pooled library transcripts revealed 26 genes differentially expressed between LMP and invasive ovarian cancers. The granulin-epithelin precursor [GEP/PC-cell derived growth factor (PCDGF)] was expressed only in the invasive ovarian cancer libraries (P < 0.028) and was absent in the LMP libraries (0 of 2872 clones). All of the invasive tumor epithelia, 20% of the LMP tumor epithelia, and all of the stroma from both subsets expressed GEP by reverse transcription-PCR. Immunohistochemical staining for GEP was diffuse and cytosolic in invasive ovarian cancer tumor cells compared with occasional, punctate, and apical staining in LMP tumor epithelia. Antisense transfection of GEP into ovarian cancer cell lines resulted in down-regulation of GEP production, reduction in cell growth (P < 0.002), decrease in the S-phase fraction (P < 0.04), and loss of density-independent growth potential (P < 0.01).

Conclusion: cDNA library preparation from microdissected tumor epithelium provided a selective advantage for the identification of growth factors for epithelial ovarian cancer. Differential granulin expression in tumor samples and the antiproliferative effects of its antisense down-regulation suggest that GEP may be a new autocrine growth factor and molecular target for epithelial ovarian cancer.

Introduction

The role of growth factors in ovarian cancer development and progression is complex and multifactorial. Growth factors identified to date, such as transforming growth factor-β (TGF-β), macrophage colony stimulating factor (m-CSF), and lysophosphatidic acid (LPA) have been shown to regulate ovarian cancer cell growth and survival in vitro and in vivo (1–4). Several of these factors may also function as biomarkers of disease or as predictors of patient outcome (1, 5, 6). The signaling pathways activated by these growth modulatory factors are now recognized as putative molecular therapeutic targets. The coupling of new high-throughput technologies with the ability to isolate specific cells from a complex microenvironment through microdissection (7) has contributed to the enlarging database of undiscovered growth factors for ovarian cancer and other cancers. We hypothesized that the identification of differences in expression between ovarian tumor cells selectively procured from invasive and noninvasive (LMP) ovarian tumors would yield novel regulatory factors for ovarian cancer.

A direct comparison of invasive and LMP tumors was chosen because of the inherent biological behavioral differences between these two classes of ovarian tumors. Although both of these tumors may shed surface implants to the omentum and other peritoneal structures (8, 9), LMP tumors are distinguished from their invasive counterparts because they lack the capacity to invade into the underlying stroma and, thus, carry a much better prognosis (10–12). The infrequency of LMP tumors and the lack of cell lines for in vitro testing have limited our ability to understand the molecular and biological differences between

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2 The abbreviations used are: LMP, low malignant potential; LCM, laser capture microdissection; GEP, granulin-epithelin precursor; PCDGF, PC-cell-derived growth factor; CGAP, cancer genome anatomy project; RT-PCR, reverse transcription-PCR; BrdUrd, bromodeoxyuridine; ER, estrogen receptor.
Materials and Methods

**Tissue Processing and cDNA Library Construction.**

Anonymized samples of seven frozen primary papillary serous ovarian tumors were received from the Cooperative Human Tissue Network (CTHN, Columbus OH) and from Dr. Andrew Berchuck (Duke University, Durham, NC; IRB-approved tissue acquisition protocol); four were invasive, three of which were advanced-stage tumors, and the other three were advanced-stage LMP tumors. Frozen sections (10 μm) were cut, stained with H&E, and subjected to LCM of the tumor epithelium as described previously (13). RNA was isolated using a RNA microisolation kit (Stratagene, La Jolla, CA), and its cDNA was amplified and packaged into pAMP-1 vector as described previously (14). The cDNA libraries were provided to the CGAP through which they were sequenced, and transcripts were grouped into UniGene clusters based on 3' homology.

CGAP Database Query and Statistical Analysis. CGAP Library UniGene clusters and sequences are available to the scientific community through the CGAP website.³ We downloaded available sequences and UniGene clusters from the libraries from the CGAP website, yielding approximately 1000 clones sequenced per library. Each UniGene cluster was assigned a hit value, defined as the number of sequenced clones that grouped into that UniGene cluster. The hit frequency is the hit value divided by the total number of UniGene clusters sequenced per library.

³ Internet address: http://cgap.nci.nih.gov.

**Table 1** Differentially expressed UniGene clusters

<table>
<thead>
<tr>
<th>UniGene</th>
<th>LMP Hit value (Freq)</th>
<th>Invasive Hit value (Freq)</th>
<th>χ² P</th>
<th>K-W P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Invasive &gt; LMP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Short-chain dehydrogenase/reductase</td>
<td>0 (0)</td>
<td>12 (0.31)</td>
<td>0.047</td>
<td>0.028</td>
</tr>
<tr>
<td>Glucosidase I</td>
<td>0 (0)</td>
<td>8 (0.2)</td>
<td>0.047</td>
<td>0.028</td>
</tr>
<tr>
<td>Hs.146199c</td>
<td>0 (0)</td>
<td>11 (0.28)</td>
<td>0.047</td>
<td>0.028</td>
</tr>
<tr>
<td>Granulin/epithelin</td>
<td>0 (0)</td>
<td>21 (0.54)</td>
<td>0.047</td>
<td>0.028</td>
</tr>
<tr>
<td>Ferritin, light polypeptide</td>
<td>3 (0.11)</td>
<td>60 (1.53)</td>
<td>0.047</td>
<td>0.034</td>
</tr>
<tr>
<td>Hs.152801c</td>
<td>5 (0.19)</td>
<td>43 (1.1)</td>
<td>0.047</td>
<td>0.034</td>
</tr>
<tr>
<td>Proteasome 25S subunit</td>
<td>11 (0.42)</td>
<td>72 (1.84)</td>
<td>0.047</td>
<td>0.034</td>
</tr>
<tr>
<td><em>Homo sapien clone DKFZp564GO22</em></td>
<td>0 (0)</td>
<td>10 (0.26)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td>Hs.108391c</td>
<td>0 (0)</td>
<td>17 (0.43)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td>Hs.133294c</td>
<td>0 (0)</td>
<td>25 (0.64)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td>Hs.133300c</td>
<td>0 (0)</td>
<td>29 (0.74)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td>Hs.145327c</td>
<td>0 (0)</td>
<td>7 (0.18)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td>Hs.163696c</td>
<td>0 (0)</td>
<td>11 (0.28)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td>Hs.182124c</td>
<td>0 (0)</td>
<td>7 (0.18)</td>
<td>0.047</td>
<td>0.079</td>
</tr>
<tr>
<td><em>Homo sapien clone 23967</em></td>
<td>3 (0.11)</td>
<td>32 (0.82)</td>
<td>0.047</td>
<td>0.14</td>
</tr>
<tr>
<td><em>Erythrocyte membrane 1 protein band 4.1-like</em></td>
<td>1 (0.04)</td>
<td>16 (0.41)</td>
<td>0.047</td>
<td>0.14</td>
</tr>
<tr>
<td>Hs.44017c</td>
<td>31 (1.17)</td>
<td>115 (2.93)</td>
<td>0.047</td>
<td>NS</td>
</tr>
<tr>
<td>Hs.153455c</td>
<td>8 (0.15)</td>
<td>27 (0.69)</td>
<td>0.047</td>
<td>NS</td>
</tr>
<tr>
<td><em>Myosin regulatory light chain</em></td>
<td>3 (0.11)</td>
<td>12 (0.31)</td>
<td>0.047</td>
<td>NS</td>
</tr>
<tr>
<td><em>Human ubiquitin carrier protein</em></td>
<td>11 (0.42)</td>
<td>177 (4.52)</td>
<td>0.047</td>
<td>NS</td>
</tr>
<tr>
<td><strong>LMP &gt; Invasive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs.157043c</td>
<td>8 (0.3)</td>
<td>1 (0.03)</td>
<td>0.008</td>
<td>0.028</td>
</tr>
<tr>
<td>KIAA0741 gene product</td>
<td>15 (0.5)</td>
<td>1 (0.03)</td>
<td>0.008</td>
<td>0.028</td>
</tr>
<tr>
<td>Ribosomal protein L38</td>
<td>29 (1.5)</td>
<td>2 (0.05)</td>
<td>0.008</td>
<td>0.039</td>
</tr>
<tr>
<td>Heat shock factor-binding protein</td>
<td>727 (27.5)</td>
<td>181 (4.6)</td>
<td>0.008</td>
<td>0.039</td>
</tr>
<tr>
<td>Hs.160743c</td>
<td>92 (3.5)</td>
<td>37 (0.9)</td>
<td>0.008</td>
<td>0.039</td>
</tr>
<tr>
<td>Hs.79691c</td>
<td>52 (1.9)</td>
<td>4 (0.1)</td>
<td>0.008</td>
<td>0.039</td>
</tr>
</tbody>
</table>

³ Hit value, the number of sequenced clones that grouped into that UniGene cluster; Freq, hit frequency = the hit value divided by the total number of UniGene clusters per library.

² K-W, Kruskal-Wallis (statistical test); NS, not significant.

* CGAP UniGene identification number for differentially expressed ESTs (http://cgap.nci.nih.gov/Tissues/LibraryFinder).
Table 2  RT-PCR for GEP in LMP and invasive ovarian cancer cryostat samples

<table>
<thead>
<tr>
<th></th>
<th>Histo-Scrapea</th>
<th>Microdissected epitheliumb</th>
<th>Microdissected stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP</td>
<td>10/10</td>
<td>2/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Invasive</td>
<td>20/20</td>
<td>20/20</td>
<td>20/20</td>
</tr>
</tbody>
</table>

a Scrape of cryostat section without microdissection.  
b \( P = 0.0002 \), Student’s t test.

tumors (all serous histology) were used to investigate GEP expression (Cooperative Human Tissue Network). LCM of ~5,000 epithelial ovarian tumor cells or stromal cells was performed per case as described previously (14). Two \( \mu l \) of the 20-\( \mu l \) first-strand reaction was amplified with GAPDH (Clontech, Palo Alto, CA) and GEP primers (sense 5’-GGAGATAT-GGCTGCTGCA-3’; antisense 5’-GGATCAAGTCAACACACACACAC-3’; Ref. 15). A 1 final 20-\( \mu l \) of PCR reaction mixture contained 200 \( \mu M \) of each dNTP, 1 x reaction mix, 20 \( \mu M \) of each primer, and 2 units of ampliTaq Gold polymerase (Perkin-Elmer, Foster City, CA). Eleven cycles of touchdown PCR (16, 17) were performed consisting of 94°C for 20 s, a one-degree decline in annealing temperature per cycle from 65°C to 55°C for 20 s, and 20 s at 72°C. Twenty-four cycles of 94°C, 55°C, and 72°C for 20 s each completed the reaction. A single reaction void of template was performed with each experiment as a negative-control. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination.

Immunohistochemistry. Thawed sections (8-\( \mu m \)) were thawed and fixed with acetone. The VECTASTAIN Elite Universal ABC Kit (Vector Laboratories, Burlingame, CA) was used per the manufacturer’s recommendations. Slides were incubated with a 1:500 dilution of anti-PCDG5 (anti-GEP) polyclonal primary antibody (18), followed by antirabbit polyclonal secondary antibody. Slides were incubated with diaminobenzidine (Roche Molecular Biochemicals, Indianapolis, IN) for 10 min and counterstained with Gill’s hematoxylin. Four different examiners reviewed staining intensity and pattern (M.B.J., J.O.B., M.R., E.C.K.).

Preparation of Antibody, Lysate, and Immunooanalysis. Polyclonal rabbit antisera were raised against keyhole limpet hemocyanin (KLH)-conjugated GEP peptide (APRWDAPLRD-PAL). Antipeptide antibodies were purified over peptide-conjugated aig1g10 columns and titered for activity by immunoblot analysis. This antibody recognizes the \( Mr \) 68,000 unglycosylated form as well as various higher-molecular-weight glycosylated forms including the predominant \( Mr \) 88,000 glycoprotein. Specificity of anti-GEP antibody for each of these bands was demonstrated by competition assays using cognate peptide. Total cell lysates were prepared from subconfluent cells using radioimmunoprecipitation assay (RIPA) buffer [50 mm Tris-HCl (pH7.6), 150 mm NaCl, 10 \( \mu g/ml \) aprotinin, 1 mm phenylmethylsulfonyl fluoride, 10 \( \mu M \) leupeptin, 2 mm Na\(_3\)VO\(_4\), 4 mm EDTA, 10 mm NaF, 10 mm sodium PP\(_3\), 1% NP40, and 0.1% sodium deoxycholate]. Protein was determined using the bicine protein determination assay (Pierce Endogen, Rockford, IL). Fifty \( \mu g \) of lysate were subjected to reducing gel electro- phoresis followed by immunoblotting with anti-GEP antibody (18). Bands were detected with enhanced chemiluminescence following standard protocols (ECL, Pierce, Rockford, IL).

Cell Culture and Transfection. SKOV3 and OVCAR3 cells were obtained from American Tissue Culture Collection (Rockville, MD). The HEY-A8 cells were a gift of Dr. Gordon Mills (M. D. Anderson Cancer Center, Houston, TX). All of the cell lines were maintained in RPMI supplemented with 10% FCS, and penicillin and streptomycin, unless otherwise indicated. A 404-bp GEP fragment (~30 to 737 bp), engineered with 5’ XbaI and 3’ SalI sites, was cloned from SKOV3 mRNA using standard PCR cloning protocols. This fragment was sequenced and subcloned in the antisense orientation into the PCNeo mammalian expression vector (Promega, Madison, WI). Either empty vector (0.5 or 1 \( \mu g \)) or antisense GEP plasmid DNA (0.5 or 1 \( \mu g \)) was transfected into HEY-A8 or OVCAR3 cells using FuGen6 transfection reagent per manufacturer’s recommendations (Roche). Transfectants were selected and maintained in 600 \( \mu g/ml \) and 1000 \( \mu g/ml \) of G418, respectively (Life Technologies, Inc., Gaithersburg, MD) and studied in proliferation assays.

Proliferation and Cloning Assays. Five hundred thousand cells, stably transfected with the empty vector control or antisense GP construct, were cultured in serum-containing medium in 24-well plates. Cell monolayers were fixed and stained at 72 h with 0.5% crystal violet in 20% methanol. Specifically bound dye was eluted with a 1:1 solution of 0.1 M sodium citrate (pH4.2)-100% ethanol, and absorbance at 540 nm was determined. Proliferation was also assessed using BrdUrd incorporation on a background of serum-limited or serum-free conditions. Twenty thousand empty vector control or antisense GEP stably transfected cells were plated in 96 wells in triplicate. Cells were serum-starved for 24 h and were replaced with either serum-free medium or 2% FCS-containing medium. Cells were labeled with BrdUrd for 2 h at 72 h, then fixed and analyzed according to the BrdUrd ELISA kit (Oncogene Research Products, Cambridge, MA). Data are reported as the mean of the absorbance measured at 450 and 595 nm per manufacturer’s instructions. Cells used for the soft agar study were subjected to transfection, harvested 48 h after transfection, and placed into 0.3% Difco agar on a bed of 0.5% agar in serum-containing medium in replicate wells (19). G418 (400 \( \mu g/ml \)) was incorporated into the agar for selection. Colonies over 50 cells (HeyA8) or 25 cells (OVCAR3) were counted at 10 days and 3 weeks, respectively. Results were compared using the Student t test, and a \( P < 0.05 \) was considered statistically significant. All three of the experiments were performed in at least triplicate.

Results

GEP Is Statistically Significantly Expressed in Invasive Ovarian Cancer Libraries. Statistical analysis of the LMP library pools against the invasive library pool identified 26 differentially expressed UniGene clusters (\( P < 0.047 \); Table 1). Of the 20 differentially expressed UniGene clusters in the invasive libraries, 42% (11 of 20) were present only in the invasive ovarian cancer libraries at this depth of sequencing, whereas the remaining 9 were more frequently expressed in invasive cancer over LMP tumors (\( P = 0.047, \chi^2 \); Table 1).

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Granulin (Grn, cluster ID = Hs.180577) had the highest hit frequency of any of the known UniGene clusters in the invasive libraries and was absent in the LMP libraries (0 of 2872 clones). This gene shares 100% identity with the human granulin precursor protein (GYHU granulin precursor, ID = pir:GYHUG) in the National Center for Biotechnology Information (NCBI) database. The granulins and epithelins are derived from the same precursor molecule and the current nomenclature, the GEP, reflects their common etiology (20, 21). Granulin expression was identified in all of the ovarian cancer libraries in the CGAP database; however, only the seven described herein were tumor epithelium specific.

**GEP Is Differentially Expressed in LMP and Ovarian Cancer Specimens.** RT-PCR done on cryostat sections were all positive for GEP transcript (Table 2). Thus, RT-PCR analysis was performed independently on microdissected tumor epithelial cells and microdissected stromal cells to differentiate the cell source. These results revealed GEP was expressed in microdissected stromal cells from all of the LMP and invasive tumors analyzed. Epithelial tumor cells from all of the invasive ovarian cancers expressed GEP (20 of 20), but in only 2 of 10 of the microdissected LMP cases did the tumor epithelium express GEP transcript. Immunohistochemical analysis was used to further evaluate GEP protein expression and cellular localization in the LMP and invasive ovarian cancer cases. GEP was detected in stroma of both LMP and invasive ovarian cancers, consistent with the gene expression results (Fig. 1). GEP staining was present in a diffuse cytosolic pattern in the epithelium of all of the invasive ovarian cancers examined. In contrast, expression of GEP was focal in LMP tumor epithelium and, where present, was punctate and apical in location. The differential expression of granulin in the libraries led to the hypothesis that GEP may be a growth factor for ovarian cancer.

**GEP Is a Growth Factor for Invasive Ovarian Cancer.**

GEP expression and its growth modulatory role in ovarian cancer cells were examined in cell lines in vitro. HEY-A8 and OVCAR3 cells were selected to test the impact of GEP on ovarian cancer cell line proliferation. HEY-A8 cells are a rap-

![GEP protein expression patterns between LMP and invasive ovarian cancer are different. GEP protein expression was demonstrated using immunohistochemical analysis of ovarian tumors with anti-GEP antibody as described in “Materials and Methods.” Stroma is positive in both LMP and invasive ovarian cancer; arrows, GEP-positive vessels. LMP tumor cells have punctate apical staining in occasional cells (A, ×100; B, ×400), whereas cytoplasm of invasive ovarian cancer tumor epithelium stain diffusely (C, ×100; D, ×400).](image_url)
GEP Stimulates Ovarian Cancer Growth

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potential and net growth rate in ovarian cancer cell lines and thus, the inhibition of GEP expression decreased proliferative cell lines (HEY-A8 60%,
and quantitated in the antisense GEP transfectants of both of the clones out readily under the G418 selection (Fig. 5). A marked reduction in density-independent cloning capacity was observed to grow more slowly, requiring as much as 50% longer culture time to come to equivalent confluence. This growth-inhibitory effect was confirmed using three growth assays and conditions. Cell growth in monolayer culture with serum-supplemented medium was diminished significantly, by up to 30% in antisense transfectants at 72 h (P < 0.002; Fig. 3). S-phase fraction was investigated with BrdUrd incorporation and was reduced to 70% (P < 0.04) in the antisense transfectants cultured in serum-free and serum-limited conditions (Fig. 4). The addition of 2% FCS to the cells in culture partially abrogated the inhibitory activity of antisense GEP. Colony formation in soft agar was studied using the introduction of freshly transfected unselected cells in the presence of G418 selection pressure. This experimental design does not rely on long-term clonal selection as is required for development of a stable transfec tant. Only cells resistant to neomycin have the capacity to clone out as shown by the lack of colonies in the FuGene transfection minus DNA controls. Neo control transfectants clon out readily under the G418 selection (Fig. 5). A marked reduction in density-independent cloning capacity was observed and quantitated in the antisense GEP transfectants of both of the cell lines (HEY-A8 60%, P < 0.02; OVCAR3 70%, P < 0.007). Thus, the inhibition of GEP expression decreased proliferative potential and net growth rate in ovarian cancer cell lines and reduced density-independent cloning capacity in soft agar.

Discussion

We hypothesized that gene expression analysis of cDNA libraries constructed from the serous ovarian tumor LMP and invasive ovarian tumor epithelium would uncover novel mediators of ovarian cancer. Analysis of cDNA libraries constructed from microdissected ovarian tumor epithelium yielded a statistically significantly increased presence of GEP transcripts in invasive ovarian cancer libraries, with no copies detected in the pooled LMP tumor libraries (0 of 2872 clones). GEP sequences are expressed in all of the invasive ovarian cancer cDNA libraries in the CGAP database. However, all other ovarian cancer libraries were constructed from bulk tissue, inclusive of stroma. Our finding of stromal GEP expression in all of the tested samples suggests that the bulk tissue libraries may be biased by inclusion of the stroma. We demonstrated that GEP is a growth factor for invasive epithelial ovarian cancer cells through down-regulation of GEP protein production by antisense transfection. Reduced GEP protein production was associated with less net-cell growth, decreased S-phase fraction, and reduced density-independent cloning capacity in soft agar. Thus, GEP, identified through a statistical analysis of expressed genes in cDNA libraries constructed from clinical tumor samples, was shown to be a biologically important and new growth factor for invasive ovarian cancer.

GEP is the largest member of a cysteine-rich family of polypeptides that include M₆, 6,000 proteins called granulins or epithelins (15, 22–25). Although initially cloned over a decade ago, characterization of this growth factor is in its early stages. Two of the smaller granulin-epithelins, granulins A and B, have been shown to have opposing effects on kidney cell growth (26), and granulin D was demonstrated to regulate human glioblastoma multiforme cell line growth in vitro (27). The granulin precursor sequence (progranulin) was originally deduced by cDNA cloning of the M₆, 6,000 polypeptides and was thought to be an inactive precursor molecule that required further processing to become functional (20). A M₆, 88,000, glycosylated form of the granulin precursor, PCDGF, was purified from the tumorigenic murine teratoma PC cell line and was shown to promote growth of this cell line in an autocrine fashion (25). This glycoprotein was also shown to regulate the growth of breast cancer cell lines both in vitro and in vivo (18, 28, 29). Increased and inducible expression of PCDGF/GEP was demonstrated in ER-positive human breast carcinoma cells when compared with immortalized nontumorigenic human mammary epithelial cells (30). PCDGF was found to be constitutively expressed in ER-negative breast cancer cells and was shown to mediate estrogen mitogenic activity and stimulation of cyclin

![Fig. 2 Transfection with antisense GEP construct decreases GEP protein production. Lysates from cells stably transfected with empty vector or antisense GEP were subjected to immunoblot for cellular GEP. The passages examined for GEP production correspond to those passages that were used for the growth assays shown in Figs. 3 and 4 and are representative of at least two replicate immunoblots for each passage. kDa, Mᵣ in thousands.](image)

![Fig. 3 Expression of antisense GEP reduces ovarian cancer cell growth under serum-containing conditions. Stably transfected cells were subjected to normal serum-containing monolayer culture conditions for 72 h before fixation and staining. Exposed dye is a measure of whole cell number and a surrogate for cellular proliferation. Data represent three independent experiments done in triplicate and are presented as mean ± SE.](image)
D1 expression in ER-positive MCF-7 cells (29). Antisense inhibition of PCDGF expression in teratocarcinoma and ER-negative breast cancer cell lines resulted in decreased cell growth in vitro and reduced the incidence and size of tumor xenografts in nude mice (18, 28, 29). The presence of GEP has been shown also in renal cell carcinoma, gastric cancers, and glioblastomas (27, 31–33). These data suggest a role for GEP/PCDGF as an autocrine growth factor in multiple tumors. In addition, GEP/PCDGF is expressed in a variety of normal rodent tissues (34) and has been shown to be present in both early and late embryonic development in a pattern suggesting a functional role for this growth factor in normal cell physiology (35, 36). Although GEP has been shown to be expressed in normal ovarian epithelial cells in one report, its biological importance in the ovarian epithelium has not been elucidated.

We identified GEP as differentially expressed between LMP and invasive ovarian cancer tumor epithelium and demonstrated that this altered expression was carried to the protein and function levels. Although GEP is both expressed and translated in the stroma of both tumors, our data suggest that the LMP tumor epithelium can either not recognize and/or not respond to stromally produced GEP or that stromal GEP may not activate the same pro-growth pathways in the LMP tumors. The presence of GEP protein in some LMP tumors, despite the lack of transcript detected in the cDNA libraries and by RT-PCR, may be explained by rare transcript abundance (less than ~1 of 3000 genes), shorter transcript half-life in LMP cells with longer protein half-life, or paracrine GEP expression by stroma with binding and internalization in the LMP tumor epithelium. Stromal expression of GEP, particularly staining of microves- sels within the stroma, leads to a secondary hypothesis in which GEP plays a role in creating a permissive host-tumor microenvironment (37). Lastly, it is possible that LMP tumors may also lack functional receptors for GEP, as yet uncloned and uncharacterized.

A functional role for GEP was shown using antisense transfection into proliferating and invasive epithelial ovarian cancer cell lines. Reduction in produced protein in the antisense cells resulted in a growth reduction that was augmented in serum-limited conditions. Even in the presence of other growth factors, such as in full or partial serum-containing conditions, the loss of GEP protein in the antisense cells resulted in a growth reduction that was augmented in serum-limited conditions. Even in the presence of other growth factors, suggesting that it may be an autocrine growth factor for ovarian cancer cells. Furthermore, down-regulation of GEP production and secretion caused a reduction in cloning capacity in soft agar in an experimental design that does not rely on long-term clonal selection as is required for development of stable transfectants. These data suggest that GEP expression provides necessary growth and survival signals for ovarian cancer cells. Supportive of this hypothesis is the reduction in activated Akt found in antisense transfectants (preliminary results). A similar end point was observed in another model system in which transfection of full-length GEP into R- mouse embryo fibroblasts, that lacked the insulin-like growth factor receptor I, allowed the cells to proliferate in the absence of other growth factors (38, 39). In addition, both GEP transfection of R- cells and PCDGF stimulation of MCF-7 human breast cancer cells activated the mitogen-activated protein (MAP) kinase pathway (29, 38).

The identification of GEP through the statistical analysis of cDNA libraries demonstrates the power of this approach to uncover differentially expressed genes from patient-derived tumor samples. Our observations were made possible by microdissection of epithelial cells selectively from the surrounding stroma, underscoring the importance of the template used for molecular analysis. The finding that GEP is present in both stroma and epithelium makes its role in tumor progression more intriguing and the need for identification, cloning, and character-ization of its receptor paramount. Our data indicate that GEP...
may be an important growth factor for the development of the clinically aggressive subtype of epithelial ovarian cancer. The multifunctional role of GEP in ovarian cancer also makes this protein an attractive molecular target for further investigation.

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