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Advances in Brief

Frequent Silencing of the *GPC3* Gene in Ovarian Cancer Cell Lines

Hsingchi Lin, Reid Huber, David Schlessinger, and Patrice J. Morin[1]

Laboratory of Biological Chemistry [H. L., P. J. M.], and Laboratory of Genetics [R. H., D. S.], Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224, and Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287 [P. J. M.]

Abstract

***GPC3* encodes a glypican integral membrane protein and is mutated in the Simpson-Golabi-Behmel syndrome. Simpson-Golabi-Behmel syndrome, an X-linked condition, is characterized by pre- and postnatal overgrowth as well as by various other abnormalities, including increased risk of embryonal tumors. The *GPC3* gene is located at Xq26, a region frequently deleted in advanced ovarian cancers. To determine whether *GPC3* is a tumor suppressor in ovarian neoplasia, we studied its expression and mutational status in 13 ovarian cancer cell lines. No mutations were found in *GPC3*, but its expression was lost in four (31%) of the cell lines analyzed. In all of the cases where *GPC3* expression was lost, the *GPC3* promoter was hypermethylated, as demonstrated by Southern analysis. Expression of *GPC3* was restored by treatment of the cells with the demethylating agent 5-aza-2′-deoxycytidine. A colony-forming assay confirmed that ectopic *GPC3* expression inhibited the growth of ovarian cancer cell lines. Our results show that *GPC3*, a gene involved in the control of organ growth, is frequently inactivated in a subset of ovarian cancers and suggest that it may function as a tumor suppressor in the ovary.**

Introduction

Twenty-five thousand new cases of ovarian cancer will be diagnosed this year in the United States, yet very little is known about the molecular determinants of this disease. Mutations in *BRCA1*, *BRCA2*, or mismatch repair genes can cause familial forms of the disease, but these syndromes affect only a small proportion of ovarian cancer patients (1). Unfortunately, the genes involved in the familial syndromes have shed little light on the mechanisms of sporadic ovarian tumorigenesis. Several chromosomal abnormalities have been identified in sporadic ovarian cancers. The extent and location of LOH[2] depend on the subtype, but high frequencies of loss are generally observed at chromosomes 17p, 17q, and 22q (2, 3). Recently, it was reported that a region located at Xq26 is frequently deleted in advanced ovarian cancer (4). *GPC3*, a gene located at Xq26, was recently shown to be mutated in Simpson-Golabi-Behmel syndrome, an overgrowth syndrome also involving multiple embryonal neoplasia (5). The *GPC3* gene occupies almost 1 Mb at Xq26 and makes this region relatively gene poor (6). *GPC3* is expressed ubiquitously in the embryo (5), but shows an expression pattern restricted to the ovary and the colon in the adult.[3] Moreover, *GPC3* expression inhibits growth of mesotheliomas and MCF-7 breast cancer cells *in vitro*, probably by inducing apoptosis (7). For these reasons, we decided to investigate *GPC3* status in ovarian cancers.

Materials and Methods

Cell Lines. Ovarian cancer cell lines ES-2, OV1063, MDAH 2774, SK-OV-3, HS571, CA-OV-3, and OVCAR-3 were obtained from the American Type Culture Collection. Cell lines AD 10, UCI101, UCI107, A222, and A224 were a gift from Dr. Michael Birrer (National Cancer Institute, Bethesda, MD). Cell line A2780 was kindly provided by Dr. V. Bohr (National Institute on Aging, Baltimore, MD). All cell lines were maintained in McCoy's 5A medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), except lines AD 10, UCI101, UCI107, A222, and A224, which were maintained in RPMI 1640 (Life Technologies, Inc.), supplemented as above.

RT-PCR and Genomic PCR. Total RNA was isolated using Trizol (Life Technologies, Inc.). Total RNA (10 μg) was used for reverse transcription using Superscript II (Life Technologies, Inc.). After synthesis, the cDNA mixture was diluted with one volume of TE [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA], and 1–2 μl of the mixture was used as PCR template. Primers GPC-1a (5′-ctcctagctccctgcgaag) and GPC-6b (5′-tctccagtacttgtcaatctc) were used to amplify the 5′ fragment of *GPC3*. The 3′ fragment was amplified using primers GPC-9a (5′-gttactgcaatgtggtcatgc) and GPC-11b (5′-acatgtgctgggcaccag). Primers bact-1a (5′-tctacaatgagctgcgtgtg) and bact-4b (5′-catctcttgctcgaagtcc) were used for amplifying β-actin as a control. GPC-EX1A (5′-aggtagctggcgaggaaac) and GPC-EX1B (5′-taggcacgctcaagggac) were used to amplify *GPC3* exon 1 using 0.1 μg of genomic DNA. PCR conditions were as described (8), using amplitaq DNA polymerase (Perkin-Elmer Corp.).

Methylation Analysis. For Southern analysis, 10 μg of genomic DNA was digested with either *Eco*RI alone or a combination of *Eag*I and *Eco*RI. The restriction digested DNA fragments were separated on a 0.8% agarose gel. After transfer, the membrane was hybridized with the radiolabeled 3′ promoter fragment amplified by PCR using primers GPC3-pr8a (5′-tgagattcagtcacagtaagg) and GPC3-pr11b (5′-ttctggattggttctcgcac). Cell lines A224, ES-2, and OV-1063 were tested for restoration of expression by demethylase treatment (9). Briefly, 50% confluent cells were treated with 0.5 μM 5-aza-2′-deoxycytidine (Sigma Chemical Co.), for either 2 or 4 days. The medium was replenished every 2 days. RNA isolation for RT-PCR was performed as described above.

Colony-forming Assay. Cells were grown to 50–70% confluence in 100-mm Petri dishes and treated with a mixture of transit-100 (Panvera Co.) and pGPC3 or pCINEO plasmid DNA in serum free Optimem I media (Life Technologies, Inc.) for 5 h. At the end of the incubation, the cells were washed with PBS and grown in the regular medium for 48 h. The cells were then split into 1:5 or 1:10 ratio grown in media containing G418 (500 μg/μl) in 100-mm Petri dishes. About 7–9 days later, cells were stained with 0.2% crystal violet in 10% ethanol, and the visible colonies were counted and compared. Colony number for vector alone was normalized to 100%.

DNA Sequencing. Two *GPC3* cDNA fragments, 5′ and 3′, were PCR amplified separately using primers GPC-1a/GPC-6b and primers GPC-9a/GPC-11b, respectively. The PCR products were gel-purified using Spin-X centrifuge filter tubes (Costar), followed by ethanol precipitation. The purified fragments were used as DNA templates for direct sequencing using Thermosequenase (Amersham Corp.) and ^{33}P-labeled ddNTPs (Amersham Corp.). PCR primers (above) and internal primers GPC-4a (5′-caagcctgactccacaagc), GPC-13a (5′-gttgctcatgtagaacatgaag) and GPC-14a (5′-aactgaagcacattaaccagc) were used for sequencing. Exon 8 of *GPC3* was amplified using primers GPC-EX8A (5′-tagtgttatactgaggctatg) and GPC-EX8B (5′-catggttagtcctctacttc) and sequenced using primer GPC-EX8A.

Results

***GPC3* Expression in Epithelial Ovarian Cancer Cell Lines.** Because *GPC3* is expressed in the normal adult ovary, we designed a RT-PCR strategy to investigate its expression in ovarian cancer cell lines. A 3′ region of the *GPC3* cDNA could be amplified from 9 of the 13 cell lines (Fig. 1), whereas 4 cell lines (ES-2, OV1063, A222, and A224) did not show any amplification. The same result was obtained when using a

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[1] To whom requests for reprints should be addressed, at Laboratory of Biological Chemistry, Gerontology Research Center, National Institute on Aging, NIH, 5600 Nathan Shock Drive, Baltimore, MD 21224. Phone: (410) 558-8506; Fax: (410) 558-8386.

[2] The abbreviations used are: LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR.

[3] R. Huber, G. Pilia, and D. Schlessinger, unpublished observations.

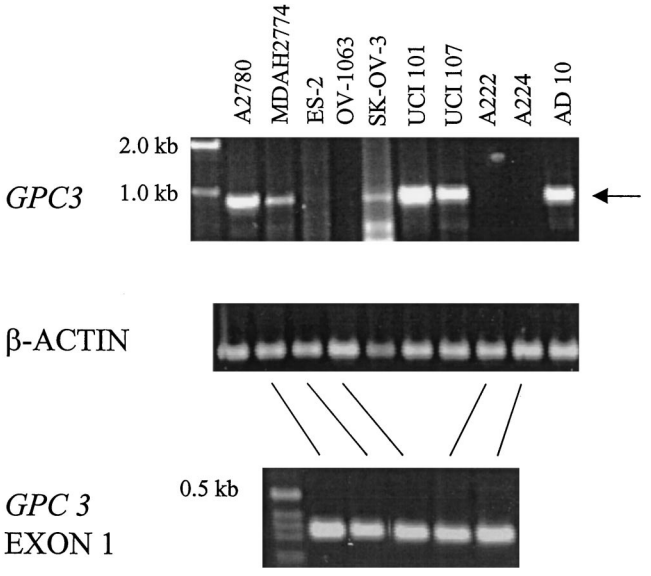

Fig. 1. *GPC3* expression in ovarian cancer cell lines. *Top*, the 0.9-kb 3′ *GPC3* fragment obtained by RT-PCR (*arrow*) in the indicated cell lines. As a positive control for cDNA integrity, a portion of *β-actin* was amplified by RT-PCR (*middle*). *Bottom*, *GPC3 exon1* as detected by genomic PCR for cell lines MDAH2774, ES-2, OV-1063, A222, and A224.

5′ RT-PCR product.[4] SK-OV-3 exhibited a smaller product (Fig. 1) that was shown, by sequencing, to be a nonspecific PCR artifact[4] As a control, a PCR product corresponding to the housekeeping gene *β-actin* was amplified from all of the lines, demonstrating the integrity of the cDNAs. Exon 1 (Fig. 1), exon 3, and exon 8[4] could be amplified from genomic DNA of all of the nonexpressing lines, verifying that the gene was not deleted or grossly rearranged.

Methylation of the *GPC3* Promoter. The results above demonstrate that *GPC3* expression is lost in several ovarian cancer cell lines. Because the *GPC3* promoter contains a CpG island (6), we investigated its methylation status in these lines. Once cleaved with *Eco*RI, the *GPC3* promoter was contained in an 8-kb genomic fragment (Fig. 2*A*). The methylation status of the promoter was investigated by codigesting the DNA with *Eag*I, a methylation-sensitive restriction enzyme. In the absence of methylation, the 8-kb DNA fragment can be cleaved by *Eag*I leading to the detection of a 4.2-kb fragment by Southern analysis. When digested with *Eco*RI and *Eag*I, cell lines A2780, MDAH2774, and SK-OV-3, which all expressed *GPC3*, exhibited the 4.2-kb fragment characteristic of the unmethylated allele (Fig. 2*B*). A2780, MDAH2774, and SK-OV-3 had a 1:1 ratio, suggesting the presence of one hypermethylated and one unmethylated allele. Interestingly, cell lines ES-2, OV1063, and A224, which were all nonexpressors, were completely methylated at the *GPC3* locus. None of the *GPC3*-expressing cell lines tested showed complete hypermethylation at the GPC3 promoter (Table 1). Cell line A222, a nonexpressor, contained a small amount of the unmethylated allele, suggesting that the population was not homogeneous, with a small proportion of cells having unmethylated *GPC3*. It should be pointed out, however, that only one *Eag*I site was investigated, and the heterogeneity might arise from nonhomogeneous methylation across the CpG island. It is also possible that A222 is aneuploid with a majority, but not all, of the alleles methylated at the *GPC3* promoter.

To determine whether the methylated, inactive alleles of *GPC3* could be reactivated, the cells lines were treated with the demethylating agent 5-aza-2′-deoxycytidine. Expression of *GPC3* could be restored in cell lines OV1063, ES-2, and A224 after 2 days of treatment (Fig. 3), consistent with the notion that methylation of the promoter was responsible for the loss of expression. SK-OV-3 exhibited a smaller nonspecific band that was shown, by sequencing, to be a nonspecific PCR artifact.[4] In addition, to confirm these results, the RT-PCR assay for the reactivation experiment was repeated with a variety of primer pairs. In all cases, reactivation of *GPC3* expression by 5-aza-2′-deoxycytidine was observed.[4]

Growth Suppression by *GPC3*. The results presented above suggest that *GPC3* expression is specifically lost during ovarian tumorigenesis. To investigate GPC3 effect on the growth of ovarian cell lines, the *GPC3* cDNA was transfected into cell lines ES-2, UCI-101, MDAH 2774, A2780, and A224. Ectopic expression of *GPC3* resulted in significant inhibition of colony-forming efficiency in cell lines ES-2 and A224, where endogenous *GPC3* expression is lost (Fig. 4 and Table 1). *GPC3* did not significantly affect colony-forming abilities of cell lines UCI101, MDAH2774, and A2780, which retained endogenous *GPC3* expression.

Sequence Analysis of *GPC3* in Ovarian Cancer Lines. Because *GPC3* expression was lost in ~30% of the cell lines analyzed, we investigated the status of *GPC3* in the cell lines where expression was detected. *GPC3* was amplified from cDNA in two separate segments and completely sequenced in cell lines SK-OV-3, MDAH2774, HS571, CA-OV-3, A2780, UCI101, UCI107, AD10, and OVCAR-3. Two unreported polymorphisms were found in cell lines A2780, UCI107, and AD10 (Table 1). In each of these three cell lines, there were two silent changes in the coding region: a T to C transition at bp number 1697 and an A to G transition at bp number 1823. Interestingly, the changes were homozygous at the cDNA level, but heterozygous at the genomic level, indicating that two alleles were present but only one was expressed. All of the other expressing cell lines were wild type for *GPC3*. OV1063, a cell line that did not express *GPC3*, contained a rare change in exon 8, corresponding to the 3′ untranslated region of the gene (Table 1). Although, this change was not encountered in any of the other lines, no wild-type sequence was found in the genomic DNA of OV1063, suggesting that this line is hemizygous for *GPC3*.

Discussion

Recently, it has become apparent that tumor suppressors can be inactivated in cancer by mutations, deletions, rearrangements, or

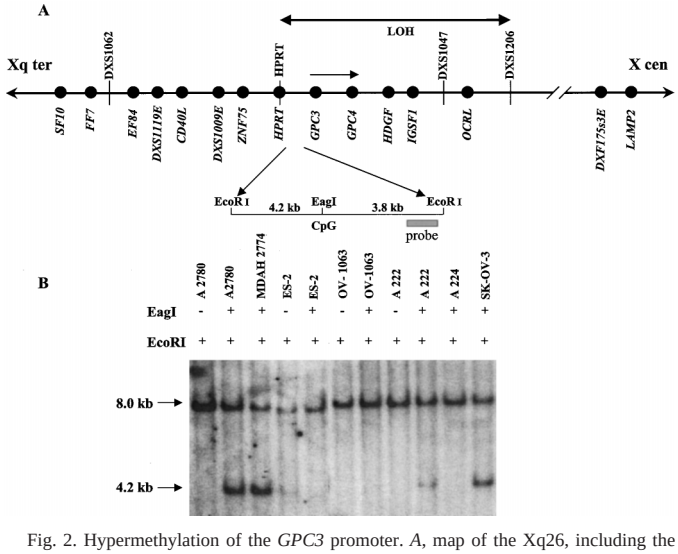

Fig. 2. Hypermethylation of the *GPC3* promoter. *A*, map of the Xq26, including the known genes. The *GPC3* promoter is shown. The *Eag*I restriction endonuclease is methylation-sensitive. *B*, the PCR product close to the 3′ end (shown in *A*) was labeled and used as a probe for Southern analysis of the methylation status of ovarian cancer lines. After *Eco*RI or *Eag*I and *Eco*RI restriction digests of genomic DNA of the indicated lines, hybridization was performed. The 8-kb band (corresponding to the methylated allele) and the 4.2-kb band (corresponding to the unmethylated allele) are shown by *arrows*.

[4] H. Lin and P. J. Morin, unpublished observations.

Table 1 *Sequence analysis and growth inhibition by* GPC3 *in ovarian cancer cell lines*

Cell lines	*GPC3* sequence	Colony-formation assay (% of reduction after pGPC3 transfection compared with control vector)	Methylation status at EagI site
A 2780	T1697->C, A1827->G[a]	13%	One methylated allele
MDAH 2774	Wt[b]	39%	One methylated allele
ES-2	No expression	79%	Complete methylation
OV-1063	C1978->T (no expression)	ND	Complete methylation
SK-OV-3	G1993->A	ND	One methylated allele
UCI 101	Wt	12%	ND
UCI 107	T1697->C, A1827->G	ND	ND
A 222	No expression	ND	Mostly methylated
A 224	G1993->A (no expression)	85%	Complete methylation
AD 10	T1697->C, A1827->G	ND	ND
HS 571	Wt	ND	ND
CA-OV-3	Wt	ND	One methylated allele
OV-CAR-3	Wt	ND	ND

[a] The nucleotide number is based on *GPC3* cDNA sequence.
[b] Wt, wild type; ND, not determined.

hypermethylation (9–12). In this study, we show that *GPC3* is silenced by hypermethylation of its promoter in 4 of 13 ovarian cancer cell lines. We believe *GPC3* to be a good candidate for being the target gene of the LOH reported on Xq26. The LOH frequency of ~30%, reported by Choi *et al.* (4), is consistent with our finding that 31% of the cell lines have silenced *GPC3*. The minimum region of loss extends ~5 cM centromeric to *HPRT* (4) and encompasses the *GPC3* locus (5). The only genes present in >2 Mb of DNA centromeric to HPRT are *GPC3* and *GPC4*, another recently characterized glypican gene.[5] *GPC4*, however, shows a ubiquitous pattern of expression in the adult. None of the other genes known to be in the region of loss are good candidates for being an ovarian tumor suppressor (Fig. 2A). HDGF is an endothelial mitogen (13), *IGSF1* is an immunoglobulin domain-containing gene highly expressed in adult testis and fetal liver (14), and *OCRL* is the gene mutated in Lowe's syndrome, also known as oculocerebrorenal syndrome (15).

A putative tumor suppressor gene on the X chromosome suggests several interesting mechanisms for tumorigenesis. It would seem unlikely to find a tumor suppressor gene on the X chromosome because men, with only one copy, would become highly susceptible to cancer compared with women. Negative growth-regulatory genes located on the X-chromosome would have to control the growth of female tissues, like the ovary.

Assuming that, in female cells, one allele of *GPC3* is inactive as a result of X-inactivation, a single hit in the active allele would be sufficient to inactivate the gene. In that case, methylation, mutation, or LOH of the active allele could all contribute to the loss of function of the tumor suppressor gene. Because we observe no mutations and because the expression of *GPC3* can be restored by demethylation, it seems that the most likely mechanisms in this case would be LOH or hypermethylation of the active allele. There is evidence that demethylation can lead to reactivation of X-inactivated genes (16). On the other hand, Choi *et al.* have shown that the loss occurs in the inactive allele and suggested that the target gene at Xq26 may escape X-inactivation (4). This seems to be in contradiction with results showing that *GPC3* is X-inactivated in female cells (17). It is possible that the *GPC3* locus becomes demethylated early in tumorigenesis, which would explain why the loss at Xq26, a late event, can be found in the inactive allele. Resolving these possibilities will require LOH and *GPC3* hypermethylation studies on a large panel of ovarian tumors. However, our finding that OV1063 contains only one, hypermethylated, allele is consistent with our hypothesis that both allelic loss and hypermethylation contribute to *GPC3* inactivation.

The phenotype of Simpson-Golabi-Behmel syndrome patients suggests that *GPC3* functions in regulating the balance between cell growth and cell death, as would be expected for a tumor suppressor gene. GPC3 belongs to a class of glypican-related integral membrane proteins that have been implicated in signal transduction (18). Interestingly, GPC3 may interact directly with IGF2 (5), a growth factor thought to be important in ovarian cancer (19). In addition, it was recently shown that *GPC3* induces apoptosis in MCF-7 cells and that it inhibits colony-forming ability in a cell line-specific manner (7). More specifically, mesothelioma cell lines and MCF-7 breast cancer cells were inhibited by *GPC3* transfections, but not colon line HT-29 or NIH 3T3 fibroblasts. This difference almost certainly reflects the differential activity of the pathway in different cells. Interestingly, mesotheliomas and ovarian cancers are related neoplasms because they both originate from the coelomic epithelial cell layer. We find that *GPC3* inhibits growth of cells that have lost endogenous expression, strongly suggesting that the loss of expression is not a random event in tumorigenesis, but a selected event that favors ovarian cancer growth. Similar arguments have been made to interpret inhibition of colony-forming ability by p53 and p16 (20, 21). *GPC3* may be involved in the regulation of apoptosis in the normal ovary. Lack of apoptosis leading to deregulated tissue homeostasis has emerged as an important mechanism for tumorigenesis (22). The data presented here suggest a new pathway for ovarian carcinogenesis and may lead to novel screening, diagnosis, and treatment strategies.

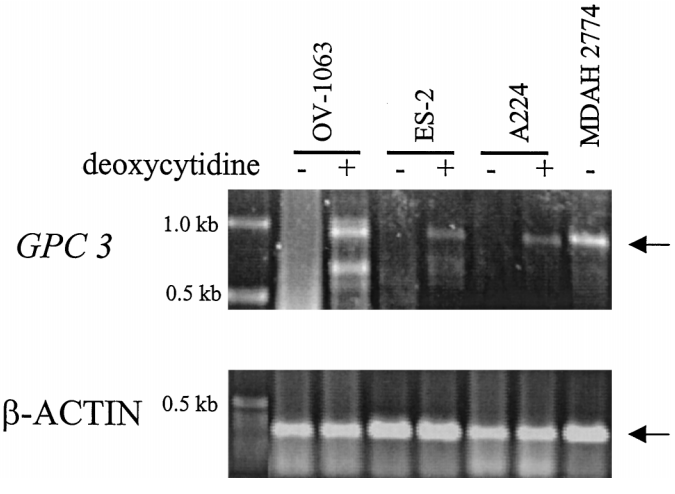

Fig. 3. Reexpression of *GPC3* after demethylation. The indicated cell lines were treated for 2 days with 0.5 μM 5-aza-2′-deoxycytidine. *GPC3* expression was monitored by RT-PCR, as described in "Materials and Methods." An *arrow* shows the 3′ *GPC3* PCR fragment. The smaller DNA fragment amplified in OV-1063 after treatment is a nonspecific PCR product, which has been confirmed by sequencing. Untreated MDAH 2774 is included as a positive control.

[5] R. Huber, R. Mazzarella, C. N. Chen, E. Chen, M. Ireland, S. Lindsay, G. Pilia, and L. Crisponi, submitted for publication.

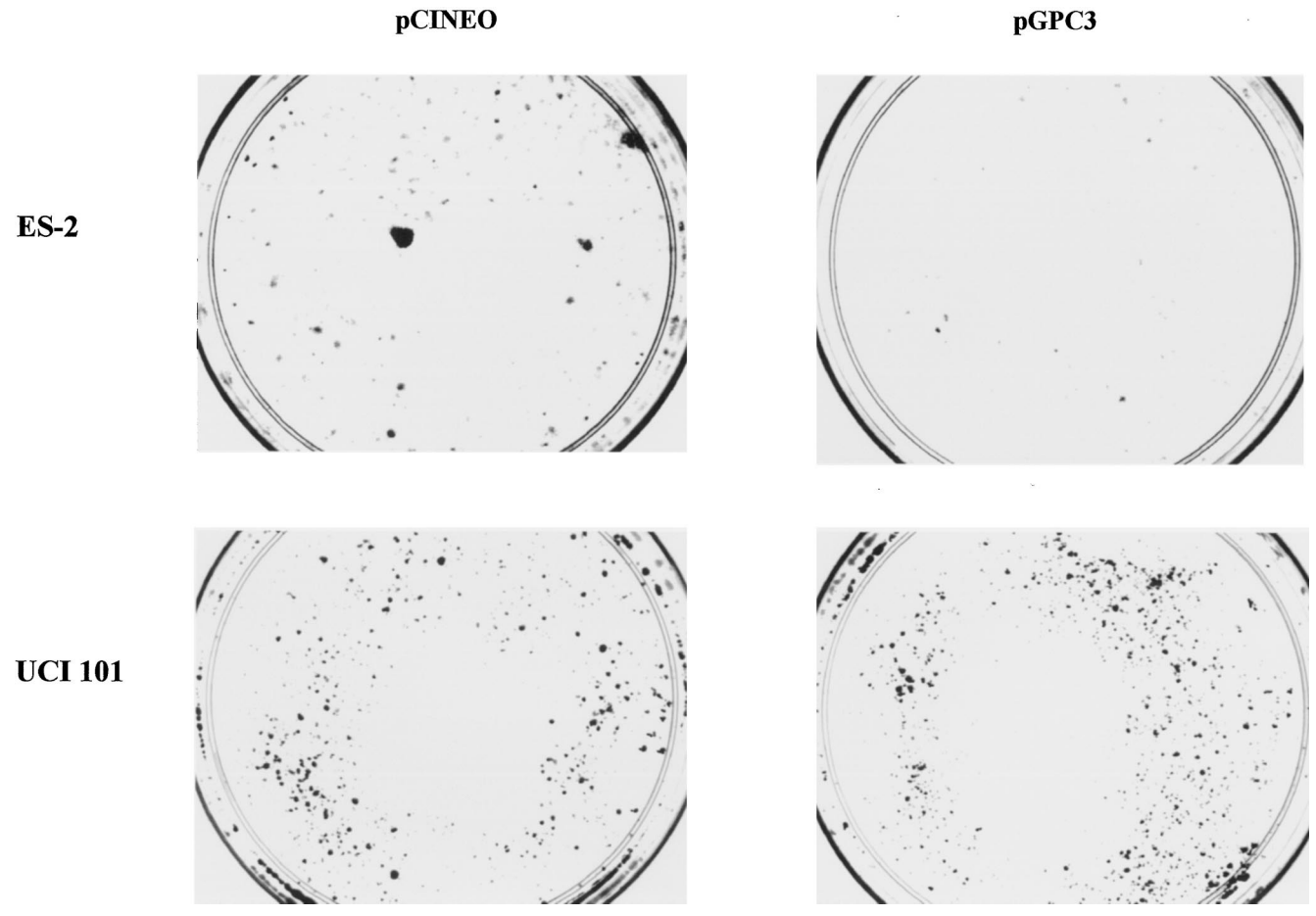

Fig. 4. Colony-forming assay. ES-2 or UCI101 cells were transfected with either the control plasmid pCINEO or a *GPC3* expression plasmid (*pGPC3*). The number of colonies was reduced 80% in ES-2 cells when transfected with the *GPC3* expression vector as compared with the pCINEO vector. The number of colonies of UCI 101, which expresses endogenous *GPC3*, was not significantly reduced by transfection with the *GPC3* expression plasmid.

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