

Choice of Normal Ovarian Control Influences Determination of Differentially Expressed Genes in Ovarian Cancer Expression Profiling Studies

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ABSTRACT

Purpose: As with many cancers thought to be of epithelial origin, expression profiling studies of ovarian cancer have relied on a variety of sources of normal cells for comparison with tumors, including whole ovary samples (WO), ovarian surface epithelium (OSE) exposed to short-term culture, and immortalized OSE cell lines (IOSE). Our purpose was to assess the impact of the use of different types of normal controls on the determination of gene expression alterations in ovarian cancer studies.

Experimental Design: We compared the gene expression profiles generated on an 11,000-element cDNA microarray of OSE brushings, whole ovary samples, short-term cultures of normal OSE, SV40 large T antigen-immortalized OSE cell lines, and telomerase-immortalized OSE cell lines. The function of the groups as normal controls was then assessed by separate comparisons of each group to a set of 24 serous ovarian carcinoma samples.

Results: The normal groups formed robust, distinct clusters in hierarchical clustering and multidimensional scaling. The Pearson correlation coefficient for all combinations of any two of the groups ranged from 0.04 to 0.54, emphasizing the disparity of the groups. In the gene lists produced by comparing each normal group with the ovarian cancer samples, the majority of genes were unique to that

normal-cancer comparison, with no gene appearing on all five lists.

Conclusions: These results suggest that the selection of a normal control to compare with epithelial ovarian cancer samples in microarray studies strongly influences the genes that are identified as differentially expressed and complicates comparison with studies using a different normal control.

INTRODUCTION

Each year, ovarian cancer claims approximately 14,000 lives in the United States, making it the most lethal of the gynecological malignancies (1). Epithelial ovarian cancer, the most common form of ovarian malignancy, is usually diagnosed at a late stage, resulting in a poor prognosis (1). The search for a better understanding of the pathogenesis of epithelial ovarian cancer and the need for tumor markers that allow early detection have led to the application of new molecular techniques such as microarray technology in ovarian cancer research.

Expression profiling studies involve the comparison of malignant ovarian cells with their normal counterpart. Choosing the appropriate source for the normal counterpart has proven difficult, however, not only for ovarian cancer but also for epithelial malignancies in general. Epithelial ovarian cancer is thought to arise from the single cell layer of OSE² (2). Because the OSE represents only a fraction of the total ovary, isolation of significant quantities of OSE RNA has been difficult. The inclusion of stroma in the normal control allows for higher RNA yields and may provide relevant information regarding gene expression alterations, given the possibility that aberrant epithelial-stromal interactions distinguish malignant tissues (3); unfortunately, it may also mask critical epithelial changes. Controversy also surrounds the decision to collect normal tissue from the same patient as the tumor or from a different patient thought to be disease free (4). The adjacent normal approach limits the inclusion of genes that are differentially expressed due to interpatient variability but have little impact on malignant transformation, whereas the normal donor approach recognizes that even normal-appearing tissue in a diseased individual may exhibit gene expression changes from truly normal tissue.

Because all of these arguments have merit, researchers have used a variety of “normal” equivalents in array studies of epithelial cancers. In the ovary, one method involves taking a portion of a normal ovary (5–8), resulting in a sample in which

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² The abbreviations used are: OSE, ovarian surface epithelium; WO, whole ovary; NOSE, cultured normal human OSE; IOSE, SV40 large T antigen-immortalized OSE; TIOSE, telomerase-immortalized OSE; CHTN, Cooperative Human Tissue Network; MDS, multidimensional scaling; hTERT, human telomerase reverse transcriptase.

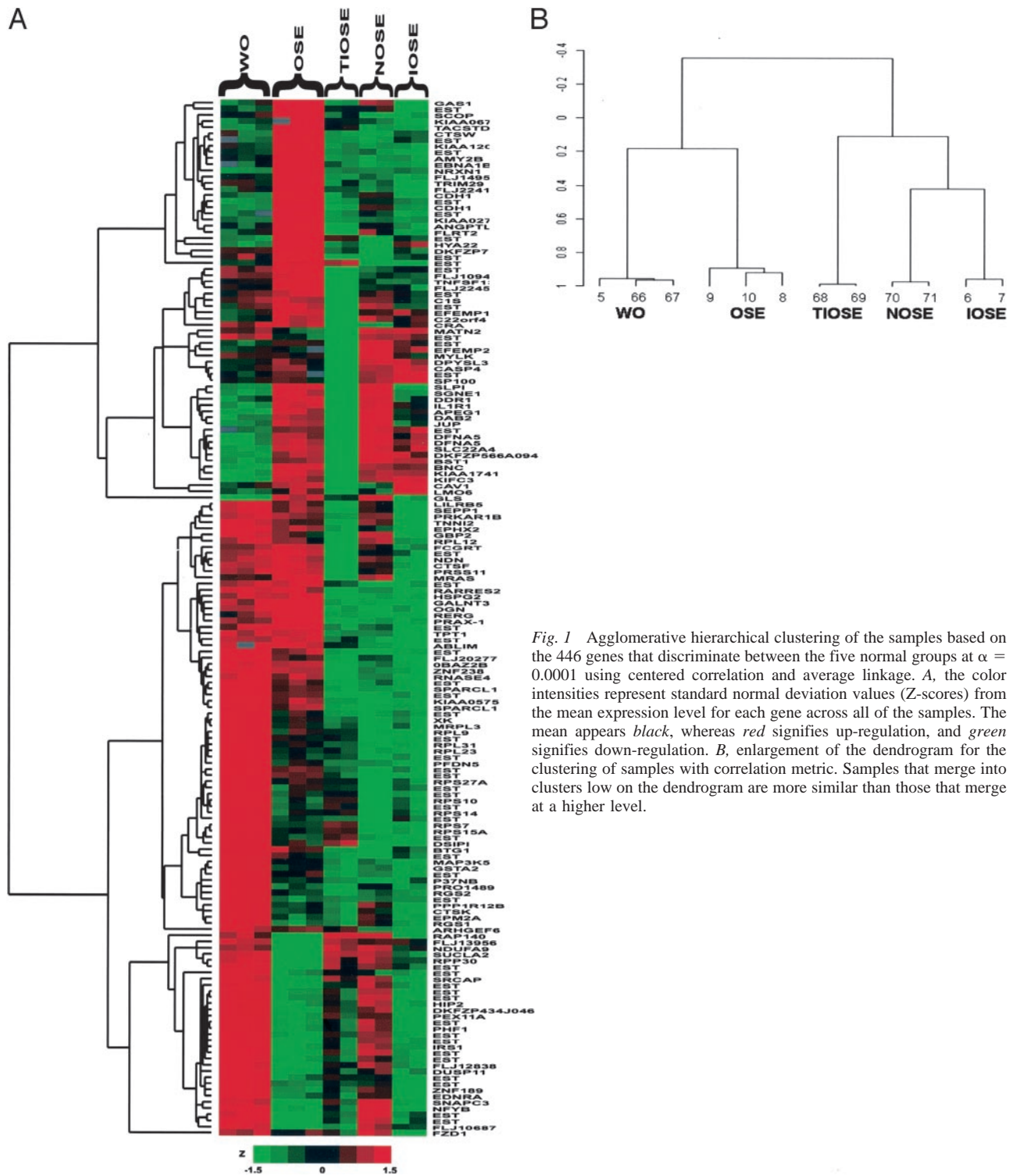


Fig. 1 Agglomerative hierarchical clustering of the samples based on the 446 genes that discriminate between the five normal groups at $\alpha = 0.0001$ using centered correlation and average linkage. A, the color intensities represent standard normal deviation values (Z-scores) from the mean expression level for each gene across all of the samples. The mean appears black, whereas red signifies up-regulation, and green signifies down-regulation. B, enlargement of the dendrogram for the clustering of samples with correlation metric. Samples that merge into clusters low on the dendrogram are more similar than those that merge at a higher level.

stroma constitutes the vast majority of the tissue. Other approaches rely on short-term cultures of OSE scrapings, which are usually termed NOSE [or HOSE (5, 9–13)]. These cultures are greatly enriched in OSE but are exposed to tissue culture

conditions. An alternative method involves NOSE cells immortalized with SV40 large T-antigen (IOSE; Ref. 14). More recently, telomerase immortalization techniques have been applied to NOSE (TIOSE), allowing their use in array

experiments.³ Finally, techniques have been developed that involve preservation of OSE brushings without culture (15); our protocol involves immediately brushing of the ovaries at the time of surgery, before manipulation can disrupt the OSE, followed by direct immersion in solution to preserve RNA quality. These cells are therefore not exposed to culture as the NOSE cells are. We compared the gene expression profiles of these five normal groups with each other; in addition, each normal group's expression profile was compared with that of a set of serous ovarian carcinomas.

MATERIALS AND METHODS

Tissue Specimens. OSE brushings were obtained with a Safetex sterile cytology brush (Hardwood Products Co., Guilford, ME) from the normal ovaries of donors during surgery for other gynecological disease at Brigham and Women's Hospital (Boston, MA) according to an institutional review board-approved protocol. Postmenopausal donors were selected because this is the group in whom ovarian cancer most frequently arises. The brush was first touched to a glass slide and then immediately placed in Trizol (Invitrogen, Carlsbad, CA). The slide was later stained following a modified Papanicolaou protocol to confirm epithelial content. Although total RNA yields are generally less than 1 µg for these brushings, pooling of samples was avoided because the amplification step in our protocol allowed a single patient's specimen to produce sufficient quantities for hybridization. Frozen WO samples were obtained from the CHTN bank (Philadelphia, PA). Pathological examination confirmed the absence of disease; as expected, the single cell layer of OSE represented only a small portion of each sample compared with stroma. Frozen samples of stage III or IV serous ovarian carcinoma were obtained from the CHTN and from patients at Memorial Sloan-Kettering Cancer Center (New York, NY) according to an institutional review board-approved protocol at the time of their initial staging operation.

Cell Lines. IOSE 80 and 120 cell lines were grown in 1:1 Media 199:MCDB 105 with gentamicin (25 µg/ml) and 2% heat-inactivated serum. NOSE cell lines were derived from OSE brushings cultured in 1:1 Media 199:MCDB 105 with gentamicin (25 µg/ml) and 15% heat-inactivated serum. RNA was extracted from cells harvested in passage 2. TIOSE cell lines were obtained from transfection of hTERT into NOSE maintained in 1:1 Media 199:MCDB 105 with gentamicin (25 µg/ml), 15% heat-inactivated serum, and G418 (500 µg/ml). Cells were cultured under standard conditions at 37°C in a humidified incubator containing 95% room air and 5% CO₂ atmosphere. RNA was extracted from cells harvested in passage 16.

RNA Amplification and Array Preparation. Total RNA was extracted from each of the normal tissues and the CHTN ovarian cancer samples using Trizol according to the manufacturer's instructions. An RNeasy kit was used to extract total RNA from the Memorial Sloan-Kettering Cancer Center ovarian cancer samples (Qiagen, Valencia, CA). RNA quality was confirmed by agarose gel electrophoresis. Universal Human

Table 1 Pearson correlation coefficient (*r*) of expression profiles between samples in a group and between groups

Group	Within group	With OSE	With WO	With IOSE	With TIOSE	With NOSE
OSE	0.78	1	0.47	0.23	0.04	0.36
WO	0.86	0.47	1	0.10	0.22	0.39
IOSE	0.73	0.23	0.10	1	0.28	0.54
TIOSE	0.93	0.04	0.22	0.28	1	0.27
NOSE	0.90	0.36	0.39	0.54	0.27	1

Reference, which includes 10 human cell lines, was used as the reference (Stratagene, La Jolla, CA). A single round of amplification of 3 µg of total RNA was performed for all samples and the reference using a modified Eberwine protocol.⁴ The fidelity of microarray results using amplified compared with total RNA has been documented, particularly for the linear amplification technique we used (16, 17). The cDNA probes were prepared according to National Cancer Institute protocol.⁴ Five µg of Cy-3-labeled reference cDNA and Cy-5-labeled sample cDNA were hybridized to an 11,000-element array produced by the National Cancer Institute Microarray Facility using Incyte UniGEM clones and ovary-specific genes from the Cancer Genome Anatomy Project. The array contains 9,896 genes, 1,306 of which represent expressed sequence tags. After approximately 16 h of hybridization, the arrays were washed and then scanned on an Axon 4000A laser scanner (Union City, CA).

Microarray Data Analysis. The BRB ArrayTools software developed by Dr. Rich Simon and Amy Peng was used for analysis.⁵ Group classification was performed by ANOVA using a stringent α to limit the number of false positive results. Multiple permutations generated a *P* to aid in assessing the reliability of the classification. An agglomerative hierarchical clustering algorithm was used in both a supervised and an unsupervised manner. Cluster reproducibility measures were calculated to assess the integrity of the clustering in the face of perturbation of the data. These measures include a robustness index (R-index), which measures the proportion of pairs of specimens within a cluster for which members of the pair remain together in the reclustered perturbed data, and a discrepancy index (D-index), which measures the number of additions or omissions when comparing an original cluster with the best-matching cluster in the reclustered perturbed data (18). The expression profiles for the samples within each group as well as the group expression averages were compared in X-Y scatterplot diagrams, generating Pearson correlation coefficients (*r*). MDS allowed assessment of the likeness of the samples' expression patterns by compressing their 11,000-dimension profiles into three-dimensional space, where samples with similar profiles cluster relatively closely.

Northern Blot Analysis. One µg of total RNA was separated on 1% agarose formaldehyde gels and transferred to Nytran SPC membranes (Schleicher & Schuell, Keene, NH). The filters were hybridized with ³²P-labeled cDNA probes

³ M. J. Birrer and S. C. Mok, unpublished data.

⁴ Internet address: <http://nciarray.nci.nih.gov/reference/index.shtml>.

⁵ Internet address: <http://linus.nci.nih.gov/BRB-ArrayTools.html>.

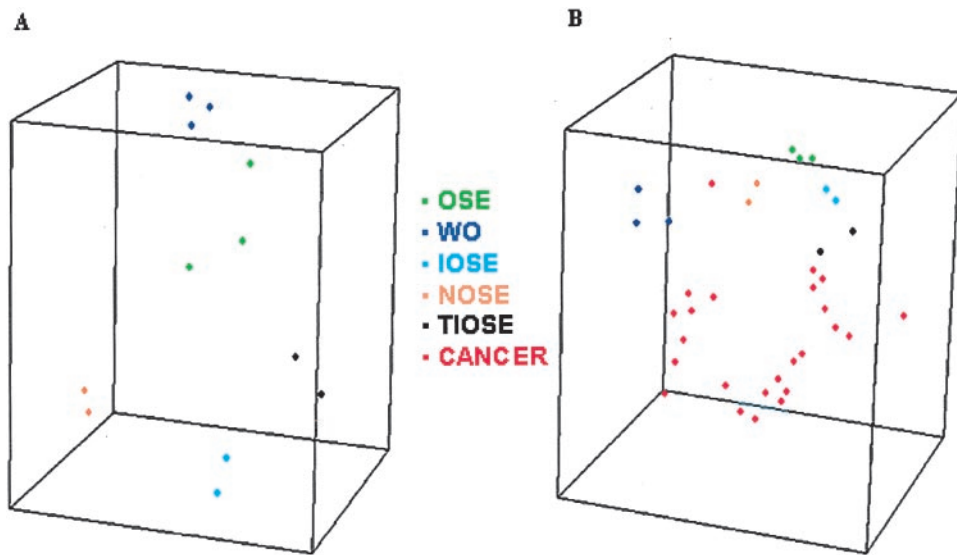


Fig. 2 MDS allowing assessment of the likeness of the samples' expression patterns by compressing their 11,000-dimensional profiles into three-dimensional space, where samples with similar profiles cluster relatively close. A, unsupervised MDS of the normal groups. B, unsupervised MDS of the normal groups and the serous ovarian carcinoma samples.

Table 2 Comparison of gene lists generated by comparing each normal group with ovarian cancer

Ovarian normal groups	No. of genes normal vs. Cancer ^a	No. of unique genes	Unique genes (%)
OSE	176	127	72
WO	118	92	78
IOSE	192	98	51
TIOSE	242	188	78
NOSE	178	113	63

^a $\alpha = 0.0001$.

Table 3 Comparison of expression data for *N-cadherin* and *aldose reductase* on microarray and Northern blot

	IOSE	TIOSE	NOSE	OSE	WO
<i>N-Cadherin</i>					
Microarray ^a	5.9	1.46	9.43	0.79	0.27
Northern ^b	8.77	1.29	5.82	0.15	0.01
<i>Aldose Reductase</i>					
Microarray	1.34	1.82	8.46	0.39	0.48
Northern	3.57	1.13	5.34	0.30	0.19

^a Expressed as geometric mean of ratio of expression for normal group compared with reference.

^b Expressed relative to 18S.

corresponding to five of the differentially expressed genes (*N-cadherin*, *aldose reductase*, *p53*, *p21*, and *p27*). The 18S band was used to control for RNA loading.

RESULTS

Comparison among Normal Groups. A classification comparison that acknowledged the presence of five groups revealed 446 genes that discriminated between them at $\alpha = 0.0001$ with a 2000 permutation $P = 0.0005$. In addition, each of the 446 genes identified had a highly significant P . These P s

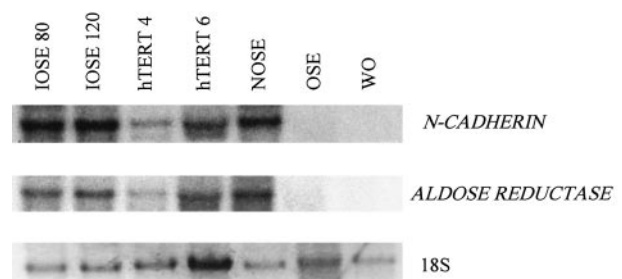


Fig. 3 Confirmation of array data with Northern blot analysis. Northern blot for *N-cadherin* and *aldose reductase*. Two samples of IOSE (IOSE 80 and 120) and TIOSE (hTERT4 and hTERT6) were included with one sample of the other types of cells.

suggest that both the ability of a set of 446 genes to distinguish the groups and the presence of each of the 446 genes on that list are unlikely to be due to chance alone. Hierarchical clustering was performed first based on all of the genes (unsupervised) and then based on the 446-gene list (supervised), revealing five clusters with R-index = 1 and D-index = 0. The indices demonstrate that the clusters were perfectly robust, with no discrepancies in sample assignment to a particular cluster in the face of slight data perturbations. The distinct profiles of the five groups are visible in the image plot produced by supervised hierarchical clustering (Fig. 1A). Two main branches occurred in the resulting dendrogram for the clustering of the samples, which normally would appear along the top of Fig. 1A but is enlarged in Fig. 1B to facilitate viewing. The first branch included the uncultured tissue samples (WO and OSE brushings), whereas the second contained the samples that were cultured (NOSE, IOSE, and TIOSE). Interestingly, further branching appeared at approximately 0.2 on the correlation metric, suggesting that each group is quite distinct. In contrast, the samples within each group approached a correlation of 1.0.

This pattern was reiterated when the gene expression pro-

Table 4 Genes up-regulated >2.5-fold in NOSE compared with OSE brushings, expressed as fold-difference in geometric mean

Gene	Fold-difference NOSE:OSE	Function (ref. no.)
<i>Glioma pathogenesis-related protein</i>	9.2	p53 regulated (21)
<i>Mesoderm-specific transcript homologue</i>	6.0	Imprinted gene
<i>Lysine hydroxylase</i>	3.8	TNF- α^a regulated (19)
<i>LEDGF</i>	3.3	Survival factor increased by cellular stress (22)
<i>Tubulin, $\alpha 1$</i>	2.6	TGF- β regulated (20)

^a TNF, tumor necrosis factor; TGF, transforming growth factor.

files were compared in scatterplot diagrams. Whereas r for the various samples within each group ranged from 0.73 to 0.93, the correlation between groups was dramatically lower, with IOSE and NOSE the most highly correlated at $r = 0.54$ (Table 1). These data suggest that there is a reproducible profile for each normal group (given the high r between samples in each group) that is distinct from the profile of the other groups (given the low r between groups).

MDS allowed the gene expression profiles to be viewed in three-dimensional space. Unsupervised MDS of the normal groups, which included all of the genes, again revealed tight clustering of the samples within groups but relative separation between the groups (Fig. 2A). Supervised MDS using the 449-gene list generated by the classification tool was essentially identical to the unsupervised version except for tighter clustering of the samples within the groups, as would be expected by selecting for the genes that define the groups (data not shown). When the serous ovarian carcinomas were included, the unsupervised MDS revealed separation between the normal groups and the cancer specimens, as would be expected (Fig. 2B). Attempts to assess which normal group was most like or unlike serous cancer by judging the relative distances between them were complicated by the heterogeneity of the cancer expression profile in both supervised and unsupervised versions, particularly when viewing the rotation of the MDS through three-dimensional space.

Comparison of Normal Groups with Cancer. Each normal ovarian group was compared with a set of advanced-stage serous ovarian carcinoma samples to assess the similarity of the genes identified as differentially expressed. At $\alpha = 0.0001$, each comparison yielded a different number of genes capable of discriminating between normal and cancer, ranging from 118 for WO to 242 for TIOSE (Table 2). Furthermore, the content of each list was strikingly different, as evidenced by the fact that no gene appeared on all five lists. Although OSE brushings and WO clustered into a separate branch on the dendrogram and had one of the higher Pearson correlation coefficients at 0.47, only eight genes were included on both the OSE brushings and WO lists. Similarly, whereas NOSE, IOSE, and TIOSE clustered closer to each other than to WO and OSE brushings, only six genes appeared on all three lists. Furthermore, NOSE and IOSE, the two groups that were the most similar according to the clustering algorithm (correlation, 0.4) and the scatterplot diagram ($r = 0.54$), produced only 47 common genes when compared with cancer. In fact, the majority of the genes present on each list were unique to the comparison of that normal group to cancer (Table 2).

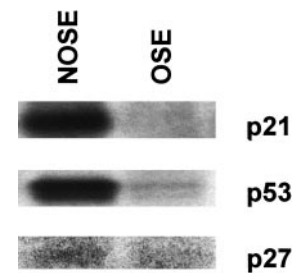


Fig. 4 Northern blot analysis of *p21*, *p53*, and *p27* expression in NOSE and OSE brushings.

Confirmation of Microarray Expression Pattern.

Northern blot analysis of two randomly selected genes found to have differential expression on microarray profiling was performed to confirm the array data. N-cadherin and aldose reductase were found to have similar patterns of increased expression in the cultured samples (IOSE, TIOSE, and NOSE) compared with the OSE brushings and WO samples on both microarray and Northern blot (Table 3; Fig. 3).

As an additional method of confirmation, we evaluated the list of known genes displaying higher expression in NOSE compared with OSE brushings. It included lysine hydroxylase and tubulin $\alpha 1$, whose expression is increased by growth factors (Table 4; Refs. 19 and 20). Glioma pathogenesis-related protein, recently identified as a p53 target with proapoptotic activity, and LEDGF, a cell survival gene with higher expression under cellular stress, also appeared on the list (21, 22). The up-regulation of these genes in NOSE suggests that the cultures were under cellular stress/growth-inhibitory conditions and undergoing cell cycle arrest. To test this, we determined the expression level of *p53*, *p21*, and *p27* genes, whose increased expression is associated with cell cycle arrest, on Northern blot. The expression of *p53* and *p21* was greatly elevated in NOSE cultures compared with OSE brushings, whereas *p27* expression was increased to a lesser extent (Fig. 4).

DISCUSSION

Microarray technology provides a powerful tool for the gene expression profiling of human cancers. As the field has evolved, investigators have realized the difficulty that using different array platforms and reference RNA samples poses for comparing results across studies. Using ovarian cancer as an example, this study indicates that the choice of a normal cell type to use as a control in epithelial cancer research is equally critical because the various control cells used to date have

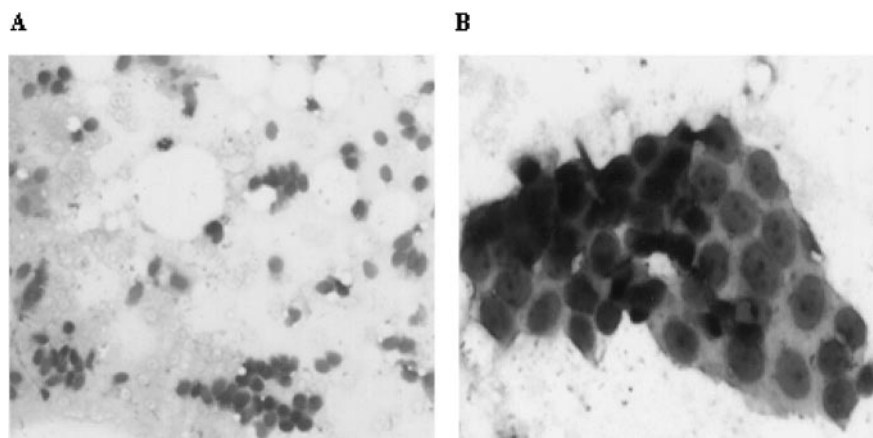


Fig. 5 Modified Papanicolaou stain of brushing from normal ovary confirming the presence of epithelial cells without significant contamination from other cell types. A, $\times 40$ magnification. B, $\times 200$ magnification.

remarkably distinct molecular profiles and identify different genes as differentially expressed when compared with ovarian cancer.

To our knowledge, this study represents the first direct comparison of the various types of normal ovarian control cells. Microarray studies of ovarian cancer generally have included one type of control cell for comparison with cancer. Primary cultures have been a popular choice due to their widespread availability, the ability to enrich epithelial content, and the ease of obtaining large amounts of RNA for microarray analysis. Concerns have arisen, however, about the degree to which cultured cells reflect their cell of origin (13, 23, 24). Our findings support these concerns in the case of OSE, showing that the primary cultures had very low levels of correlation with OSE brushings and WO.

Exposing cells to tissue culture conditions significantly alters gene expression, either by directly affecting transcriptional regulation or by selecting for a subset of cells that are not representative of the original culture. Previous work by other laboratories has demonstrated that these cultures have exquisite sensitivity to a variety of growth factors such as transforming growth factor β that diminishes as the cells become transformed (25–27). Our results support these findings because two of the most highly up-regulated genes in NOSE compared with OSE brushings are regulated by growth factors (19, 20). *LEDGF*, a survival gene induced by cell stress, is a transcription factor whose targets include heat shock proteins (22). Its up-regulation suggests that although these cultures are proliferating, many of the cells are undergoing cell cycle arrest, apoptosis, or senescence. Our demonstration of the increased expression of *p53*, *p21*, and *p27* in the primary cultures of OSE supports this idea. Presumably, the expression of some genes is dramatically altered as the cultures approach their pre-crisis stage, even in these cultures harvested in the second passage. These changes in the expression profile resulting from exposure to tissue culture conditions may complicate identification of a truly malignant, rather than a simply proliferative, pattern.

For IOSE and TIOSE cultures, the immortalization technique may result in changes to the expression profile in addition to those caused by the culture process itself (28–30). A recent proteomic analysis of prostate cells found that whereas uncul-

tured normal prostate epithelium differed only slightly from uncultured malignant epithelium from the same patient, immortalized benign and malignant cell lines from that patient had substantially different protein profiles (24). Because profiles of the primary cultures before immortalization were not included, these differences could have been due to culture, immortalization, or both. In an evaluation of NOSE cell lines by comparative genomic hybridization, however, researchers found a large increase in chromosomal imbalance after immortalization (31), suggesting that immortalization may cause abnormalities distinct from those attributable to culture conditions. Work in our laboratory supports the presence of chromosomal instability in telomerase-immortalized ovarian epithelial cells.³ In this study, the fact that the genetic profiles of OSE immortalized by either SV40 large T-antigen or hTERT are distinct from those of both OSE brushings and NOSE suggests that these cell lines have undergone unique molecular events in addition to serum exposure. Changes in the expression of genes associated with proliferation and transformation in a “normal” control are problematic for studies attempting to identify genes associated with ovarian cancer.

Taken together, the emerging data on cultured cells, whether immortalized or not, suggest that their use in studies whose fundamental aim is to identify the genes that distinguish normal from malignant ovary requires careful interpretation. Projects attempting to discover tumorigenic pathways or better tumor markers, for example, should include independent validation of expression patterns. Studies whose aim is to establish overall molecular profiles, such as a comparison of tumor profiles with clinical outcomes, would be less affected by these concerns because referring to a normal control is unnecessary. A recent study by Cho *et al.* (32) illustrates this concept well; pairwise comparisons of the genetic profiles of four histological subtypes of ovarian cancer were made, obviating the need for a normal control.

WO samples offer the advantages of relative ease of obtaining large amounts of RNA and avoidance of exposure to culture conditions. However, the presence of large amounts of stroma has the potential to obscure epithelial patterns. Proteomic data from microdissected prostate showed that <45% of proteins were shared between epithelial and stromal components

(24). In this study, the WO samples initially cluster in a branch with the OSE brushings, whereas the cultured groups form a separate branch, underscoring the importance of cell culture in expression profiles. The WO samples then form a cluster distinct from the brushings at a low correlation level, likely due to the large stromal component in the WO profile compared with the OSE brushings. The brushing technique allows the collection of OSE without stroma, which is confirmed by the presence of epithelial cells without significant contamination on the stained slide (Fig. 5). The brushings provide a relatively pure sample of OSE that is not exposed to culture conditions. As a result, in our opinion, these samples represent the most straightforward collection technique of the tissue felt to be the most biologically relevant to the development of epithelial ovarian cancer. Whereas this does not ensure that there are no alterations induced by the brushing itself, we feel that the OSE brushings are currently the best choice for a normal control in our ovarian cancer expression profiling studies. Comparison with the other types of normal controls undoubtedly will continue to offer valuable insights in ovarian cancer research, but the exact type of normal tissue used must be clearly specified because it will impact the interpretation of the results and the ability to make comparisons with studies with different normal controls.

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