Proteome Changes in Ovarian Epithelial Cells Derived from Women with BRCA1 Mutations and Family Histories of Cancer*  
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Malignant transformation of the ovarian surface epithelium (OSE) accounts for most ovarian carcinoma. Detection of preneoplastic changes in the OSE leading to overt malignancy is important in prevention and management of ovarian cancer. We identified OSE proteins with altered expression derived from women with a family history (FH) of ovarian and/or breast cancer and mutations in the BRCA1 tumor suppressor gene. Proteins from SV-40-transformed FH-OSE cell lines and control OSE lines derived from women without such histories (non-family history) were separated by two-dimensional PAGE. Gels were analyzed, a protein data base was created, and proteins were characterized according to their molecular weight, isoelectric point, and relative abundance. Mass spectrometry was performed on tryptic protein digests, and data bases were searched for known proteins with the same theoretical tryptic peptide masses. Several proteins showed altered expression in the FH-OSE cells. β-Tubulin and to a lesser extent ubiquitin carboxyl-terminal hydrolase and glyoxalase 1 appeared to be up-regulated. In contrast, proteins suppressed in FH lines include the 27-kDa heat shock protein, translationally controlled tumor protein, and several proteins associated with actin modification such as actin prepeptide, F-actin capping protein α subunit, and cofilin. Sequencing of several cofilin gel spots revealed phosphorylation of serine 3, a post-translational modification associated with decreased actin binding and cytoskeletal reorganization. Two-dimensional Western blots probed with cofilin antibody showed multiple protein spots with isoelectric points of 6–9 pH units. Blots of one-dimensional gels showed a significant reduction in cofilin expression in three FH lines when compared with three non-family history lines (p ≤ 0.05). Identification of these and other OSE proteins may be useful in detecting changes suggestive of increased risk of developing preneoplastic disease and defining the possible role(s) of the BRCA1 gene in regulation of OSE cell function. Molecular & Cellular Proteomics 4:156–168, 2005.

Ovarian cancer is the second most common cancer of the reproductive system in women and the leading cause of death from gynecological cancers (1,2). In adult women, the ovary is covered by a single layer of surface epithelial cells (OSE). Although this comprises only a small fraction of the total ovarian mass, it is the source of the majority of ovarian cancer (2). Most women with ovarian cancers have no, or nonspecific, symptoms until the latter stages of cancer development. The overall survival rate of women with ovarian cancer remains low as the majority of women are diagnosed after the disease has spread beyond the ovary. Therefore, early detection of abnormal changes in the OSE that can progress to malignancy is crucial in the prevention, surveillance, and management of ovarian cancer. This is essential for women who are at high risk for ovarian carcinogenesis due to an inherited disposition (1,3).

Overtly normal OSE cells from women with family histories of ovarian and breast cancer differ both phenotypically and genetically from OSE cells from women without such histories. OSE cells in primary culture are epithelial but usually change to mesenchymal morphology with passage (4). Such epitheliomesenchymal conversion is characterized by loss of epithelial markers such as CA125 and keratin, loss of intercellular contacts, expression of collagen, secretion of extracellular matrix, and contraction in collagenous matrix (4,5). FH-OSE cell lines often express E-cadherin (6) and retain their epithelial phenotype when compared with NFH-OSE lines (4–6). Furthermore neoplastic progression is often seen in OSE cells that fail to show epitheliomesenchymal conversion

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1 The abbreviations used are: OSE, ovarian surface epithelium; BRCA1, breast cancer antigen 1; FH, family history; NHF, non-family history; del, deletion; ex, exon; ins, insertion; HIO, history immortalized ovarian; IOSE, immortalized ovarian surface epithelial; 1-D, one-dimensional; 2-D, two-dimensional; ACN, acetonitrile; IPG, immobilized pH gradient; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; nano-LC-ESI, nanospray ionization liquid chromatography; HPLC, high pressure liquid chromatography.
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(for a review, see Ref. 2). In this study we examined the proteomes of both FH- and NFH-OSE cell lines to help elucidate signals that may be involved in their failure to convert to the mesenchymal phenotype and possibly associated with early stages of neoplastic progression.

Early onset familial ovarian cancer is often associated with loss of heterozygosity localized to chromosome 17q (7) and germ line mutations in the breast cancer antigen-1 (BRCA1) gene (8). BRCA genes appear to conform to the classic role of tumor suppressor genes as one defective copy in the germ line is sufficient to cause cancer predisposition and loss of the second allele in established tumor cells (9). The biological function of the BRCA proteins appears to be complex as these proteins are associated with a variety of cellular processes such as DNA repair and recombination, transcription, and cell cycle control (for a review, see Ref. 10). Apparently BRCA1 is essential for the error-free repair of double-stranded DNA breaks by recombination between homologous DNA sequences (10, 11). BRCA1 is phosphorylated prior to activation, binding, and regulation of other proteins involved in alteration of DNA topology, transcription, and ubiquitination. Furthermore BRCA1 plays a probable role in controlling several checkpoints within the cell cycle (10). Although cells containing a mutant BRCA1 gene show signs that p53-dependent cell cycle arrest is activated, these cells often acquire additional mutations in the p53 gene itself resulting in transformation and tumor progression (10, 12).

In this study we tested the hypothesis that the BRCA1 mutations in OSE cells lead to genetic instability that is manifested by alterations in the cell cycle and the expression of several proteins including cytoskeletal components. An unstable cell phenotype subsequently develops that is at risk for further abnormalities including tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**OSE Cell Lines and Culture Conditions—**OSE cell lines, originally derived from the ovarian surface epithelial cells from women with and without strong family histories of breast and ovarian cancer (13) at the Fox Chase Cancer Center, were immortalized by transformation with SV-40 large T antigen as described previously (14). The FH lines (HIO-104, HIO-108, and HIO-121) were tested for mutations in the BRCA1 gene (3, 13). The type and location of the mutations varied, but all three led to a truncated BRCA1 protein. HIO-104 contains one of the two Ashkenazi Jewish founder mutations in BRCA1 (185delAG).

Thus, at nucleotide 185 (in exon 2 of the BRCA1 gene), two bases (AG) are deleted resulting in a frameshift mutation and a truncated protein. The HIO106 cell line has a 1008-base pair deletion in exon 17 of the BRCA1 gene (1008bpdeleX17), and the HIO121 cell line has an insertion of 6 kilobases in exon 13 of the BRCA1 gene (ins6kbex13). Three control, SV-40-immortalized OSE cell lines (IOSE-80, IOSE-120, and IOSE-144) were derived from women without a family history of ovarian or breast cancer and were originally obtained from normal ovaries at the time of surgery for nonmalignant gynecologic diseases. Established cell lines were passaged in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 µg/ml streptomycin, 20 µg/ml gentamycin, and 1 µg/ml amphotericin B.

**Cell Lysates and Two-dimensional (2-D) PAGE—**Cells were grown to 90% confluence and washed three times with cold phosphate-buffered saline, and lysates were prepared as described previously (15, 16). Proteins were separated by their isoelectric points (pi) and relative mobility in polyacrylamide and SDS by the method of Garrels (17) as modified by Qorg et al. (18) and by our laboratory. Briefly immobilized pH gradient (IPG) strips (18 cm, Immobiline pH 4–7 or 3–10 linear DryStrips, Amersham Biosciences, Piscataway, NJ) were rehydrated with whole cell lysates containing 200–400 µg of protein and focused to 3500 V-h over a period of 20 h using a Multiphore II electrophoresis apparatus (Amersham Biosciences). IPG strips were equilibrated and fixed on top of 1.5-mm-thick slab gels consisting of 10% polyacrylamide (Duracryl, Genomic Solutions, Inc., Ann Arbor, MI). Proteins were separated by electrophoresis in SDS at a maximum of 16 watts/gel and 500 V with cooling at 10 °C using a DALT gel electrophoresis system (Amersham Biosciences).

**MALDI-MS-compatible Silver Stain and 2-D Gel Analysis—**Gels were stained using the PlusOne silver stain kit (Amersham Biosciences) with the following modifications: the glutaraldehyde was omitted from the sensitization step, and the storage buffer consisted of 10% methanol in double distilled water.

Silver-stained gels were examined visually and then scanned using an Image Acquisition Sharp JX330 scanner (Sharp Electronics Corp., Mahwah, NJ) interfaced to a SUN Sparc 4 computer (Sun Microsystems, Mountain View, CA). Images of the gels and an OSE data base was created using BioImage 2-D Analyzer software (Genomic Solutions, Inc.) on the SUN work station. Protein spots were detected, integrated intensities were measured, and relative pl values and molecular masses were assigned based on the mobility of eight selected internal standard proteins within the linear pH gradients and results from respective mass spectrometry analysis. Gels were matched and normalized for protein load, and the data base was queried for the existence of matched protein spots and differential expression among FH- and NFH-OSE proteins.

**Trypsin Digest and Mass Spectrometry—**Protein spots of interest from 2-D polyacrylamide gels were digested according to the procedure described by Rosenfeld et al. (19) as modified by Clauser et al. (20) and by our laboratory. Briefly protein spots were excised from silver-stained gels and minced with a scalpel, and the gel pieces were washed with 25 mM NH₄HCO₃ in 50% ACN, dried in a Speedvac, rehydrated in 25 mM NH₄HCO₃ solution containing trypsin (10 ng/ml, Promega, Madison, WI), and digested overnight at 37 °C. Peptides were extracted by washing twice with 50% ACN, 1% formic acid. Supernatants were concentrated by lyophilization, and the formic acid was omitted from the sensitization step, and the storage buffer consisted of 10% methanol in double distilled water.

Silver-stained gels were examined visually and then scanned using an Image Acquisition Sharp JX330 scanner (Sharp Electronics Corp., Mahwah, NJ) interfaced to a SUN Sparc 4 computer (Sun Microsystems, Mountain View, CA). Images of the gels and an OSE data base was created using BioImage 2-D Analyzer software (Genomic Solutions, Inc.) on the SUN work station. Protein spots were detected, integrated intensities were measured, and relative pl values and molecular masses were assigned based on the mobility of eight selected internal standard proteins within the linear pH gradients and results from respective mass spectrometry analysis. Gels were matched and normalized for protein load, and the data base was queried for the existence of matched protein spots and differential expression among FH- and NFH-OSE proteins.

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dependent acquisition mode. The most abundant ion of each survey scan was selected as the precursor unless otherwise specified in the exclusion list (containing commonly observed background ions and trypsin autolysis products). Collision-induced dissociation data were searched using MASCOT (the latest version of the National Center for Biotechnology Information non-redundant (NCBI nr) protein data base). Some peptides were sequenced manually, and those fragment ions were submitted for data base searching using MASCOT and MS-Tag for protein identification (Ref. 21, prospector.ucsf.edu).

RESULTS

Detection of Differentially Expressed OSE Proteins—Protein lysates from FH- and NFH-OSE cell lines were subjected to 2-D PAGE. Gels were silver-stained and computer-analyzed. Fig. 1 shows representative 2-D gels of OSE proteins isolated from the FH HIO-104 and the NFH IOSE-80 cell lines. The total number of protein spots detected in each gel varied from four to five thousand with approximately a third of them matched to all gels. Eight standard protein spots that are present in all 2-D gels of all cell lines were selected to establish MS identification protocols and facilitate computer-assisted gel matching and assignment of pI values and molecular masses. Furthermore three proteins were found to be consistently up-regulated and eight proteins were down-regulated in the FH lines when compared with the NFH-OSE lines. Also shown in Fig. 1 are the gel locations of these 11 differentially expressed and the eight standard, cellular protein spots. Protein characteristics such as pI, molecular mass, mean integrated intensity, and -fold change in expression of the differentially expressed proteins are shown in Table I.

Identification of Differentially Expressed OSE Proteins by MALDI-TOF MS—Silver-stained protein spots of interest from 2-D gels were excised, digested with trypsin, and subjected to MALDI-TOF MS and data base search analysis using the
MS-Fit search engine (Ref. 21, prospector.ucsf.edu). MALDI-TOF peptide masses of standard and differentially expressed OSE proteins and their most probable identities are listed in Tables II and III, respectively. The number of monoisotopic masses matched varied considerably with respect to each protein, covering 15–36% of the amino acid sequence in reliable matches (within 100 ppm). MALDI-TOF spectra of 4.d, a down-regulated protein in FH-OSE lines, are shown in Fig. 2. With this protein digest, substantially more ions of greater intensity were found when 2,5-dihydroxybenzoic acid was used as the matrix when compared with /H9251-cyano-4-hydroxycinnamic acid. Data base search analysis established the probable identity of this protein as translationally controlled tumor protein. Similarly the probable identity of 3.d was established as F-actin capping protein and that of 7.d was established as tropomodulin 3. Unfortunately protein digests of 1.d yielded no specific

### Table I

| Spot | pI/kDa | Protein (accession number) | Average integrated intensity | -Fold change |
|------|-------|---------------------------|----------------------------|-------------|
| 1.d  | 6.7/19| Cofilin (Swiss-Prot P23528) | 5.3                       | 16.0        | 3.0 |
| 2.d  | 5.2/49| Actin prepeptide (NCBI 178067) | 1.5                       | 3.2         | 2.1 |
| 3.d  | 5.5/35| F-actin capping protein α subunit (Swiss-Prot P52907) | 1.6                       | 2.2         | 1.4 |
| 4.d  | 5.0/23| Translationally controlled tumor protein (Swiss-Prot P13693) | 3.2                       | 5.6         | 1.8 |
| 6.d  | 5.8/28| 27-kDa heat shock protein (Swiss-Prot P04792) | 2.1                       | 3.6         | 1.7 |
| 7.d  | 5.1/36| Tropomodulin 3 (Swiss-Prot Q9NYL9) | 1.2                       | 2.0         | 1.7 |
| 8.d  | 5.6/34| Cytosolic inorganic pyrophosphatase (Swiss-Prot Q15181) | 1.8                       | 3.6         | 2.0 |
| 9.d  | 6.9/50| 3-Phosphoglycerate dehydrogenase (Swiss-Prot O43175) | 1.5                       | 3.2         | 2.1 |

### Table II

| Spot | pI/kDa | Protein identification (accession number) | Monoisotopic masses matched | Δ ppm mean ± S.D. |
|------|-------|------------------------------------------|-----------------------------|-----------------|
| ST1  | 4.8/78| 78-kDa glucose-regulated protein precursor (NCBI 6470153) | 997.51, 1153.61, 1191.63, 1210.58 | -4.20 ± 2.72 |
| ST2  | 4.5/65| Thyroid-binding protein (NCBI 339647) | 1002.50, 1002.55, 1038.55, 1066.51 | -4.57 ± 18.5   |
| ST3  | 4.4/20| Proteasome ζ chain (NCBI 8394072) | 1063.52, 1431.75, 1447.76, 1961.97 | 8.23 ± 6.15    |
| ST4  | 5.5/19| Ubiquitin carboxyl-terminal hydrolase | 886.48, 1467.76, 1679.75, 1696.95 | 12.1 ± 41.8    |
| ST5  | 5.8/57| Protein-disulfide isomerase | 877.48, 1172.54, 1341.68, 1370.69 | -4.19 ± 2.74   |
| ST6  | 6.2/22| 27-kDa heat shock protein | 1712.78, 1895.91, 2976.46 | 2016.06 ± 641.75 |
| ST7  | 6.0/12| Nucleoside-diphosphate kinase | 1515.75, 1652.76, 1664.75, 1680.75 | 2575.30 ± 562.75 |
| ST8  | 6.8/69| γ-Aminobutyric acid transaminase | 2703.39, 831.49, 960.50, 1163.62 | 1905.95 ± 59.83 |

### MALDI-TOF peptide masses of OSE protein standards

MS-Fit search engine (Ref. 21, prospector.ucsf.edu). MALDI-TOF peptide masses of standard and differentially expressed OSE proteins and their most probable identities are listed in Tables II and III, respectively. The number of monoisotopic masses matched varied considerably with respect to each protein, covering 15–36% of the amino acid sequence in reliable matches (within 100 ppm). MALDI-TOF spectra of 4.d, a down-regulated protein in FH-OSE lines, are shown in Fig. 2. With this protein digest, substantially more ions of greater intensity were found when 2,5-dihydroxybenzoic acid was used as the matrix when compared with α-cyano-4-hydroxycinnamic acid. Data base search analysis established the probable identity of this protein as translationally controlled tumor protein. Similarly the probable identity of 3.d was established as F-actin capping protein and that of 7.d was established as tropomodulin 3. Unfortunately protein digests of 1.d yielded no specific
**Fig. 2. MALDI-TOF spectra of in-gel tryptic digest of FH-OSE down-regulated spot 4.d.** Molecular masses of peptide were determined by analyzing one-tenth of the tryptic digests by MALDI-MS using Voyager DESTR operated in reflectron mode. For sample preparation, unseparated digests were mixed at a 1:1 ratio with matrix: a, 2,5-dihydroxy-benzoic acid (10 mg/ml in 20% ACN, 1% trifluoroacetic acid); b, saturated α-cyano-4-hydroxycinnamic acid in 50% ACN, 1% trifluoroacetic acid. All spectra were internally calibrated using known trypsin autolysis products at m/z 842.51 and 2211.10 (T).

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### TABLE III

| Spot | pI/kDa | Protein (accession number) | MALDI-TOF MS | ESI-LC-MS/MS | Sequence coverage % |
|------|-------|---------------------------|--------------|--------------|---------------------|
|      |       |                           | Peptide      |              |                     |
|      |       |                           | matches      | Δ ppm mean ± S.D. | Peptide sequences |                     |
| 1.d  | 6.7/19| Cofilin (Swiss-Prot P23528) | 0            |              | 3 AcApSGVAVSDBGVIK HELQANCYEEVKDR YALYDATYETK AVFPSIVGRPR | 22                   |
| 2.d  | 5.2/49| Actin prepeptide (NCBI 178067) (Swiss-Prot P52907) | 4 1.05 ± 21.6 |              | 1 VKPFMTGAAEQIK VKPFMoxTGAEEQIK | 15                   |
| 3.d  | 5.5/35| F-actin capping protein α subunit (Swiss-Prot P13693) | 22 4.00 ± 14.7 |              | 2 YALYDATYETK AVFPSIVGRPR | 36                   |
| 4.d  | 5.0/23| Translationally controlled tumor protein (Swiss-Prot Q04760) | 5 17.1 ± 10.8 |              | 3 VLGMTLQQQ FAWLALSRE GFGHIAIVPDVYSAK | 31                   |
| 5.d  | 5.1/23| Glyoxalase 1 (Swiss-Prot Q04760) | 12 13.3 ± 25.0 |              | 2 VLGMTLQQQ FAWLALSRE GFGHIAIVPDVYSAK | 23                   |
| 6.d  | 5.8/28| 27-kDa heat shock protein isoform (Swiss-Prot P04792) | 3 −57.2 ± 87.7 |              | 2 VLGMTLQQQ FAWLALSRE GFGHIAIVPDVYSAK | 20                   |
| 7.d  | 5.1/36| Tropomodulin 3 (Swiss-Prot Q9NL9) | 11 20.1 ± 19.3 |              | 1 TPVKPFMTGAAEQIK TPVKPFMoxTGAEEQIK | 32                   |
| 8.d  | 5.6/34| Cytosolic inorganic pyrophosphatase (Swiss-Prot Q15181) | 9 −7.61 ± 17.0 |              | 2 VLGMTLQQQ FAWLALSRE GFGHIAIVPDVYSAK | 25                   |
| 9.d  | 6.9/50| 3-Phosphoglycerate dehydrogenase (Swiss-Prot Q043175) | 11 −9.84 ± 14.2 |              | 1 TPVKPFMTGAAEQIK TPVKPFMoxTGAEEQIK | 17                   |
| 1.u  | 4.7/50| β-Tubulin isoform (Swiss-Prot P05218) | 5 13.3 ± 20.3 |              | 2 VLGMTLQQQ FAWLALSRE GFGHIAIVPDVYSAK | 34                   |
| ST4  | 5.6/26| Ubiquitin carboxyl-terminal hydrolase (Swiss-Prot P09936) | 5 12.1 ± 41.8 |              | 2 VLGMTLQQQ FAWLALSRE GFGHIAIVPDVYSAK | 26                   |

*a* Modifications: Ac, acetyl; pS, phosphoserine; Mox, oxidized methionine.
MALDI peptide ions when analyzed with either matrix. Nano-LC-ESI MS/MS Identification of Differentially Expressed OSE Proteins—Peptide sequence data were used to confirm the protein identity of MALDI-TOF-predicted proteins. Nano-LC-ESI MS/MS analysis and peptide sequence information derived from the MS/MS data were used to search the

(a) Nano–LC-ESI MS/MS spectrum of a peptide with m/z 718.50+2

(b) Peptide and Sequence Coverage Map (Swiss Prot: P13693)

MALDI peptide ions when analyzed with either matrix.

Fig. 3. MS/MS spectra of FH-OSE down-regulated spot 4.d. a, nano-LC-ESI MS/MS spectra of a peptide with m/z 718.50+2 (MALDI-TOF peptide MH+ 1435.70) from an in-gel tryptic digest of 4.d. Data were obtained using a PE Sciex QSTAR quadrupole time-of-flight mass spectrometer. M(ox), oxidized methionine. b, sequence and peptide coverage map. Sequences in outline correspond to peptides sequenced by nano-LC-ESI MS/MS, and underlined sequences correspond to matched peptides obtained by MALDI-TOF MS analysis.
data base using MASCOT and the MS-Tag search algorithm (Ref. 21, prospector.ucsf.edu). Fig. 3 shows the spectrum of fragment ions of a tryptic peptide from protein spot 4.d. Data base search analysis confirmed the identity of this FH down-regulated protein as translationally controlled tumor protein. Nano-LC-ESI MS/MS data combined with five experimentally derived peptide masses obtained by MALDI-TOF analysis covered 31% of the protein. The identity of those proteins whose digests failed to generate MALDI-TOF ions was also established using nano-LC-ESI MS/MS analysis (Table III). The spectra of fragment ions from one of three tryptic peptides obtained from the digest of FH down-regulated protein spot 1.d is shown in Fig. 4. Sequence data matched to the amino-terminal tryptic peptide of cofilin. Fragment ions revealed acetylation of the amino-terminal alanine and phosphorylation of serine 3, a post-translational modification associated with decreased actin binding and cytoskeletal reorganization (22–24).
Western Blot Analysis of hsp27 and Cofilin in OSE Cell Lines—Two of the identified differentially expressed proteins, hsp27 (6.d) and cofilin (1.d), are known to exist in multiple phosphorylated states, which can correspond to multiple spots on 2-D gels. In fact, MALDI-TOF peptide masses of both protein spots ST6 and 6.d matched reliably to hsp27. Therefore we examined the overall amount of both hsp27 and cofilin by probing Western blots of 1-D PAGE-separated proteins with specific antibodies. No significant difference in overall hsp27 expression was found, although one isoform of hsp27 (6.d) was consistently down-regulated in FH lines on 2-D gels. Another isoform of hsp27 that was consistently up-regulated in FH lines and that compensated for the decreased expression of protein spot 6.d was not found. There was a significant difference (p < 0.05) in the overall expression of cofilin in all three FH-OSE cell lines when compared with cofilin expression in the three NFH-OSE lines (Fig. 5). The mean integrated intensity of specific FH bands (0.63) was 1.8-fold lower than that of NFH bands (1.12). 2-D Western blots probed with specific antiserum to cofilin showed multiple protein spots of similar molecular mass but less abundant and greater pi (Fig. 6). Nano-LC-ESI MS/MS analysis con-

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2 L. Zeng and D. M. Smith-Beckerman, unpublished data.
We confirmed the identity of these proteins as cofilin. We probed 2-D Western blots from gels with an IPG pH range of both 4–7 and 3–10 with an antiserum to cofilin because the phosphorylated isoform (which migrates to a more acidic isoelectric point than the non-phosphorylated isoform) migrated to the basic edge of the IPG 4–7 pH gel. The purpose of these additional immunoblots was to pick up any additional cofilin isoforms (e.g. non-phosphorylated) that could not be seen on the original IPG 4–7 pH gels. We did not sequence many proteins from IPG 3–10 pH gels as multiple proteins were often found in the same gel location. Protein identification was more difficult, and data were not as reliable.

**DISCUSSION**

The use of mass spectrometry and data base matching algorithms to study and identify proteomic patterns associated with pathology or risk of disease has gained momentum due to its mass accuracy, speed of analysis, and efficiency of comparing peptide mass and sequence information to extensive computer protein data bases. In this study we compared the proteome of OSE cell lines derived from women with strong histories of familial breast and/or ovarian cancer and mutations in the \textit{BRCA1} gene to the proteome of OSE cell lines derived from women without such histories and known mutations. The use of 2-D SDS-PAGE and computer-assisted gel analysis coupled with in-gel digestion and mass spectrometry permitted the isolation and identification of several proteins whose expression was repeatedly altered in FH-OSE cell lines. We were able to identify three proteins that were up-regulated and eight proteins that were down-regulated in FH-OSE cells when compared with NFH-OSE cells (Fig. 1 and Table I).

The use of 2-D gels to isolate cellular proteins will exclude very large and small proteins and peptides that may represent fragments of larger proteins. Although we detected several thousand protein spots in each gel, only about a third of these could be matched across all gels. The large variation in matched spots could arise in part from genetic differences among the original human donors. It was not our intent to identify the entire proteome of OSE cells but rather to focus on differences between OSE proteins isolated from women with known mutations in the \textit{BRCA1} gene and strong family histories of breast and ovarian cancer and OSE proteins isolated from women without such histories. It is also possible that additional proteins of lesser abundance will show altered expression in FH-OSE cells when compared with NFH-OSE cell lines, but these proteins are below the level of detection of silver-stained proteins from whole cell lysates on 2-D gels. Such proteins may be detectable following enrichment proto-
cols that select for organelle-specific proteins. Thus, the changes in the proteome that we saw may represent changes in only a subset of proteins, possibly reflecting the downstream consequences of cells with genetic instability and cell cycle alterations.

MALDI-TOF data were generated using two matrices: 2,5-dihydroxybenzonic acid and α-cyano-4-hydroxycinamic acid. We found that 2,5-dihydroxybenzonic acid appeared to be superior to α-cyano-4-hydroxycinamic acid in generating ionized peptides from some protein tryptic digests such as translationally controlled tumor protein (Fig. 2). Furthermore digests of some proteins present in relatively high abundance on 2-D gels, such as cofilin, produced few or no ions with either matrix. For these proteins we had greater success with electrospray ionization and obtained sequence fragmentation ions on the QSTAR mass spectrometer that permitted reliable identification of the differentially expressed protein. Nano-LC-ESI MS/MS not only gave us the amino acid sequence of three cofilin peptides but also identified a phosphorylation of serine at residue 3 on cofilin (Fig. 4) and at residue 82 on hsp27 (protein spot 6.d).

Many of the proteins with altered expression in FH-OSE lines are structural or associated with modification of cytoskeletal proteins. Tropomodulin 3, actin prepeptide, F-actin capping protein α subunit, translationally controlled tumor protein, and cofilin are all suppressed in FH-OSE relative to their expression in NFH-OSE. While these proteins do not represent new and novel molecules that are unique to FH-OSE lines with mutations in BRCA1, they do present a consistent pattern of expression that might be useful in identifying OSE cells from women at risk of developing ovarian cancer. Why the expression of this group of cytoskeletal proteins appears altered is not immediately clear. Decreased expression of F-actin capping protein, actin prepeptide, and hsp27 has also been seen in cultured dermal fibroblasts isolated from older healthy individuals when compared with those isolated from teenagers (25). This suggests that changes in the proteome found in FH-OSE parallels some of the changes seen in aging, and consequently these lines may be at higher risk of additional genetic instability. The finding that these cytoskeletal proteins are down-regulated in FH-OSE lines relative to NFH lines is consistent with the slower growth pattern and maintained cobble stone appearance of FH-OSE cell monolayers in culture (5). In contrast, NFH-OSE cells modulate to a fibroblast-like morphology when they reach confluence. This modulation from an epithelial to fibroblastic phenotype most probably involves the remodeling of cytoskeletal proteins and microfilament assembly, which may be suppressed in FH-OSE cells.

Translationally controlled tumor protein is a highly conserved growth-related protein that has been found in a wide variety of normal and tumor cells alike and is thought to fulfill a housekeeping function (26). Among other activities, it binds to tubulin and is responsible for alterations in cell morphology (27). In fact overexpression of this protein is correlated with microtubule rearrangements seen as an increase in microtubule mass and stability. Thus, it is not unexpected that in our FH-OSE cell lines this protein is suppressed 1.8-fold when compared with expression in the NFH-OSE lines (Table I). Furthermore this alteration is consistent with our hypothesis that BRCA1 mutations in OSE cells may result in genetic instability that is manifested by alterations in the cell cycle and the expression of cytoskeletal proteins. This may lead to an unstable cell phenotype with an additional risk for further abnormalities including tumorigenesis. Also of interest is the work of Chung et al. (28) that suggests deregulation of translationally controlled tumor protein may be associated with tumor development in colon carcinoma.

A significant reduction (3-fold) in the amount of a cofilin isotype was found in FH-OSE when compared with NFH-OSE cells (Table I). In addition, a generalized reduction in cofilin expression in FH-OSE cells was also found (Fig. 5). Cofilin is normally responsible for the rapid recycling of actin monomers associated with membrane ruffling and cytokinesis (24). It is also required for regulated changes in cell shape, organelle and ion transport, and receptor-mediated responses to external stimuli. Its activity and ability to bind actin monomers is regulated by phosphorylation on serine 3, which we were able to detect by nano-LC-ESI MS/MS of protein spot 1.d (Fig. 4). Protein spots on 2-D immunoblots probed with cofilin-specific antibodies that migrate to a pi of greater than 6.7 (Fig. 6) are consistent with cofilin isotypes with reduced phosphorylated amino acid residues. Apparently dephosphorylation of serine 3 is required for binding to F- and G-actin and nuclear translocation (22–24). Therefore it is thought that cycles of phosphorylation and dephosphorylation may regulate microfilament turnover, and phosphorylation of cofilin may represent a compensating homeostatic mechanism (29).

Furthermore the cellular concentration of cofilin and other microfilament-modifying proteins may ultimately control the concentration and function of many cytoskeletal proteins including the expression of cell phenotype. With the use of cDNA arrays to study the process of oncogenesis, cofilin has also been cited as one of a group of novel potential markers of cancer (30). In addition, elevated amounts of this protein are associated with proliferation, differentiation, and loss of a tumorigenic phenotype in various tumor cells lines such as the colorectal Caco-2 line (31). Our finding that the amount of cofilin is altered in FH-OSE cell lines versus NFH-OSE lines indicates that this process may occur very early before tumorigenesis is actually observed in cells isolated from women with elevated risks of developing ovarian or breast cancer due to a family history and mutations in the BRCA1 gene. It is of interest that heterozygosity for BRCA mutations predispose women to breast and ovarian cancer (32), suggesting that exposure to hormones, growth factors, or other environmental conditions may play a role as well.

Since two isoforms of hsp27 were identified, one of which
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was suppressed in FH-OSE cells, we looked for other isoforms that may have been up-regulated to compensate for spot 6.d and to account for our failure to find a difference in total hsp27 expression between FH and NFH lines on 1-D immunoblots. Although additional hsp27 spots existed, none were consistently up-regulated more than 1.4-fold. This could be explained in part by the fact that protein spot 6.d is ~10-fold lower in abundance than protein spot ST6 (Fig. 1). Global proteomic analysis of other cell types indicates that there are many isoforms of hsp27 and that the number may be higher in malignant than in normal cells (33).

We are not the only research group that has found a relationship between BRCA1 and hsp27 expression. In cloned human prostate (DU-145) and breast (MCF-7) cancer cells as well as in mouse embryo fibroblasts, overexpression of wild type BRCA1 proteins induced the expression of hsp27 and resistance to heat sensitivity to a greater extent than did mutant BRCA1 proteins (34). Furthermore elevated culture temperatures up-regulated hsp27 but down-regulated BRCA1 proteins. Cancer cells that develop from the OSE do not necessarily contain mutant BRCA1 proteins (1–3). Consequently data showing the expression of hsp27 and its prognostic role in ovarian carcinoma may be inconsistent. In several human carcinomas, hsp27 expression has been associated with resistance to chemotherapeutic agents such as cisplatin and doxorubicin (35) and development of multidrug resistance (36). This suggests that its expression might be related to a worse prognosis. Other studies (37, 38) that monitored immunohistochemical staining of tumor tissue for hsp27 found its expression and decreased tumor growth to be independent predictors of survival. Decreased hsp27 expression was related to decreased survival over a period of 2 (37) and 5 years (38). In the current study, our use of proteomic tools such as 2-D SDS-PAGE and mass spectrometry led to the discovery that one less abundant, phosphorylated isoform of hsp27 is down-regulated in the FH lines (deficient in BRCA1 protein), yet other hsp27 isoforms of greater abundance (i.e. ST6) appear unaffected. This observation supports the theory that phosphorylation, not overall expression, of hsp27 may be most important for the pathophysiologic stress response. This may also explain, in part, the discrepancies of hsp27 expression in established ovarian carcinoma.

The ubiquitin-proteasome degradation pathway is vital for protein remodeling and several cell functions, for example, gene transcription and cell cycle regulation. Another function attributed to BRCA1 is that of a ubiquitin ligase (10) when associated with BARD1, a protein that regulates RNA processing following DNA damage (39). Thus, in cells with mutations in BRCA1, fewer proteins may be ubiquitinated and marked for degradation, resulting in the apparent abnormal expression of a variety of protein substrates. In our studies, however, we found a small but significant increase in the abundance of ubiquitin carboxyl-terminal hydrolase in the FH-OSE cell lines when compared with NFH-OSE cell lines. It is possible that this variation in the FH-OSE cells represents an imbalance among functional proteins within the ubiquitin-proteasome degradation pathway or a compensatory mechanism to rapidly process and remodel those proteins that become ubiquitinated by other mechanisms. This response may not be unique to FH-OSE cells as alterations in the ubiquitin-proteasome degradation pathway have been seen in a variety of pathologic disorders. For example, proteomic analysis of cardiac tissue from animal models and human hearts with dilated cardiomyopathy also show elevated concentrations of ubiquitin carboxyl-terminal hydrolase (40). Furthermore altered expression of ubiquitin carboxyl-terminal hydrolase isoforms has been seen in a variety of neurologic pathologies including Parkinson’s disease (41) and spinal cord injuries (42).

The application of proteomic tools to distinguish protein profiles of normal and pathologic conditions, including ovarian carcinoma, is becoming more credible. Jones et al. (43) used 2-D SDS-PAGE and mass spectrometric methods to distinguish between protein profiles of invasive ovarian cancers and noninvasive ovarian tumors with low malignant potential. The results indicate that the proteome undergoes dynamic changes over the course of the disease. Of interest is that some of the proteins that were elevated in our FH-OSE cells (for example, glyoxalase) are also overexpressed in invasive tumors (43). Therefore it is likely that a series of different proteomic patterns exist for increased risk and early and invasive stages of ovarian cancer. The challenge is to recognize the spectrum of pattern changes, use them to obtain predictive information that compliments our present diagnostic methods, and follow up with appropriate care. Accordingly Petricoin et al. (44) used surface-enhanced laser desorption ionization mass spectrometry and serum proteomic pattern analysis to discriminate patients with early and late stage ovarian carcinoma from normal individuals and those with benign disorders. Although the identities of the discriminating low molecular weight, hydrophobic serum proteins found in their study have not been identified, these peptides may be derived from the ovary or the carcinoma or consist of metabolic fragments of host response proteins. Furthermore some of these fragments may represent degradation products of a few of the proteins with altered expression that we found in FH-OSE cells relative to NFH-OSE cells. Also of interest are additional studies in which proteomic analysis of serum samples indicates that hsp27 may be a biomarker for breast cancer (45).

Our study used proteomic techniques to define a small subset of proteins with altered expression in FH-OSE cells with mutations in the BRCA1 gene as compared with that in NFH-OSE cells. For women with an increased risk of developing ovarian cancer, it is important to define proteomic changes that distinguish them from the rest of the population at the very early stages of tumorigenesis. Only further studies will determine whether changes in the proteome profiles de-
ected in very early stages of ovarian disease reflect some of the differences that we saw between our FH- and NFH-OSE cell lines.

Acknowledgments—We thank Lucy Zeng, Glenn Dawes, Jay Aquino, and Vanisree Battar of San Francisco State University for SDS-PAGE technical assistance and David Maltby of the University of California, San Francisco (UCSF) Facility for Mass Spectrometry for assistance in MS spectra collection. We also thank Drs. Susan Fisher, Steven Hall, and H. Ewa Witokowska of the UCSF Biological Resource Center Mass Spectrometry Facility for helpful discussions.

*This work was supported in part by National Institutes of Health (NIH) Research Infrastructure in Minority Institutions (RIMI) Grant 5P20RR11805 (to D. M. S.-B.) and by the San Francisco State University. Additional funds from NIH National Center for Research Resources (NCRR) Grant RR01614 supported K. E. W. as a postdoctoral fellow in the Burlingame laboratory and the University of California, San Francisco Facility for Mass Spectrometry (to A. L. B., Facility Director). NIH NCRR Grants RR01614 and RR12961 (to A. L. B.) permitted the purchase and operation of the Voyager DESTR and QSTAR Pulsar mass spectrometers in the UCSF Facility for Mass Spectrometry. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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