Location of BRCA1 in Human Breast and Ovarian Cancer Cells

In their recent report, Yumay Chen *et al.* present data suggesting that the BRCA1 gene product is excluded from the nucleus in sporadic breast and ovarian cancer and in certain breast and ovarian cancer cell lines (*1*). Immunostaining with a single, nonaffinity purified polyclonal antibody served as the criterion for establishing the subcellular localization of the protein. Here, we describe results that differ substantially from those of Chen *et al.* (*1*).

We have developed several BRCA1-specific antibodies. With the use of a BRCA1 carboxyl-terminal peptide (CQELDTYLIPQIPHSHY) as immunogen, we raised a rabbit polyclonal antiserum. It was then affinity-purified against the aforementioned peptide immunogen affixed to agarose beads (Pierce, Rockford, Illinois). We also generated a panel of monoclonal antibodies (mAbs) to BRCA1 by immunizing mice with the same peptide or with defined segments of BRCA1 that had been encoded by elements of cloned, human BRCA1 complementary DNA (cDNA) (*2*). The latter were synthesized as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli*.

We validated the specificity of these antibodies in three ways: (i) by immunoprecipitation (IP) of intact BRCA1 (≃220 kD) present in ^{35}S-methionine–labeled whole-cell lysates; (ii) by performing a protein immunoblot of unlabeled whole-cell lysates; and (iii) by IP of BRCA1 from unlabeled whole-cell lysates, followed by a protein immunoblot, with the use of antibodies to distinct epitopes of BRCA1 to immunoprecipitate the protein (Fig. 1). Endogenous BRCA1, immunoprecipitated by these antibodies, migrated as a doublet of about 220 kD in 6% SDS-polyacrylamide gels, the lower band of which co-migrated with in vitro–translated BRCA1 (Fig. 1).

Immunostaining of neutral paraformaldehyde-fixed cell lines with BRCA1 antibodies gave rise to a nuclear signal (Fig. 2). In particular, the affinity-purified rabbit polyclonal antiserum raised against the carboxyl-terminal peptide of BRCA1, and each of seven different mAbs, all produced a nuclear dot pattern. Two-color confocal immunofluorescence studies using this affinity-purified rabbit polyclonal antiserum in combination with each of the seven mAbs demonstrated co-localization of the nuclear dot pattern in each case (Fig. 2). Thus, all antibodies appeared to react with a common structure or structures.

The BRCA1 nuclear dot pattern was observed in all human cell lines examined, regardless of the tissue of origin, as well as in primary human diploid fibroblasts, primary human mammary epithelial cells (HMECs), and in all (six) breast and (six) ovarian cancer cell lines tested. Furthermore, both polyclonal antiserum and mAb elicited the same co-localizing nuclear dot immunofluorescence pattern in cells fixed with neutral paraformaldehyde, or methanol, or 70% ethanol. Thus, the nuclear dot distribution of BRCA1 is a general cellular characteristic and not the result of a fixation artifact.

In a further effort to learn whether the nuclear staining observed with the various

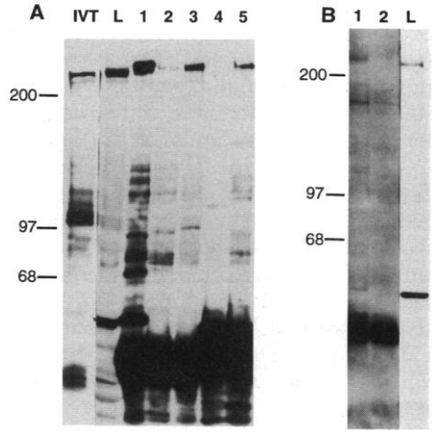

Fig. 1. Panel of antibodies that react with endogenous BRCA1 protein. Lysates of the human breast cancer cell line MCF7 (~2×10^7 cells per lane) were subjected to IP. In each case, a different monospecific antibody to BRCA1 was used. Immunoprecipitates were dissolved, separated in a 6% SDS-polyacrylamide gel, and a protein immunoblot for BRCA1 was performed (*5*). BRCA1 migrated above the 200-kD molecular weight marker. Lanes in which an immunoprecipitation step was performed contain an intensely staining immunoglobulin heavy chain signal (resulting from cross-reaction with the secondary antibody) that migrated to the lower molecular weight regions of the gel. Lanes labeled "IVT" and "L" were not preceded by an immunoprecipitation step. (**A**) IP performed with mAbs to BRCA1. IPs in lanes 1 to 5 were performed with the addition of 5 μl of affinity-purified rabbit polyclonal antibody to mouse IgG (Cappel, West Chester, Pennsylvania). IVT, 15 μl of rabbit reticulocyte lysate containing in vitro–translated, clonal human BRCA1 was analyzed, as a control. The 220-kD band seen in this lane was shown by autoradiography to be labeled by ^{35}S-methionine. In vitro translation performed in the absence of clonal BRCA1 did not synthesize ^{35}S-labeled proteins migrating in this region of the gel; thus, the 220-kD band detected after in vitro translation of clonal BRCA1 is presumed to be a BRCA1 gene product. L, MCF7 whole-cell lysate (50 μg of total protein); lane 1, IP with mAb MS110 (*2*); lane 2, IP with mAb MS13 (*2*); lane 3, IP with mAb AP16 (*2*); lane 4, IP with mAb GG22 (negative control, raised against human E2F4); and lane 5, IP with SG11 (*2*). This gel is presented as a composite, as the in vitro translate (IVT) immunoblot signal was stronger than other lanes at equivalent exposures. (**B**) IP using A19, an affinity-purified rabbit polyclonal antiserum to the BRCA1 carboxyl-terminal peptide CQELDTYLIPQIPHSHY. Lane 1, IP with A19; lane 2, IP with A19 that has been preincubated with a 20-fold molar excess of the immunizing peptide; L, MCF7 whole-cell lysate (50 μg of total protein). This gel is presented as a composite, as the signal from the whole-cell lysate immunoblot (L) was stronger than that of the A19 IP at equivalent exposures. Efficiency of immunoprecipitation by some BRCA1 antibodies appeared to be improved by the presence of small quantities of SDS and deoxycholate in the lysis buffer.

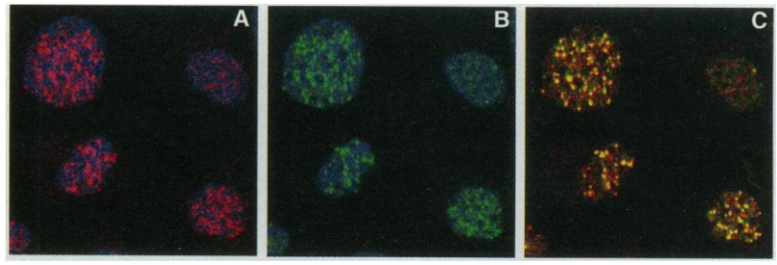

Fig. 2. BRCA1 localizes to a nuclear structure or structures. Cell lines were cultivated on glass cover slips, fixed in neutral paraformaldehyde, and then Triton-permeablized as described previously (*3*). Cover slips were stained with antibodies to BRCA1 with A19 (rhodamine/red) and mAb MS13 [fluorescein isothiocyanate (FITC)/green], were counterstained for DNA (DAP/blue), and observed with confocal microscopy. Antibodies were incubated at 37°C. Image shows ovarian cancer cell line, SKOV-3. (**A**) Image showing A19 + 4,6-diamidino-2-phenylindole (DAPI) stains. A19 stain produces a nuclear dot pattern. (**B**) Image showing MS13 + DAPI stains. MS13 stain produces a nuclear dot pattern. (**C**) Conjoint image showing the additive effect of the A19 + MS13 stains. Yellow signal occurs where A19 (red) and MS13 (green) stains overlap, indicating the localization of the BRCA1 protein. Negative controls yielded no significant nuclear signal. Similar BRCA1-specific nuclear dot patterns were seen in AX11, AT14, SW626, OVC-1, and CAOV (ovarian cancer cell lines); in MCF7, T47D, MDAMB435S, MDAMB415, MDAMB157, and MDAMB231 (breast cancer cell lines); and in U2OS, HeLa, 293, DU145, SAOS-2, IMR90 (primary human diploid fibroblasts), and HMEC. Antibodies elicited a nuclear dot pattern in CV1 and COS cells, indicating extensive sharing of epitopes between human and monkey BRCA1.

antibodies depends on special cell fixation conditions, we tested the subcellular localization of BRCA1 by biochemical extraction analysis in unfixed cells. Three cancer cell lines (MCF7, SKOV-3, and U20S), each characterized by dotlike nuclear staining and the absence of cytoplasmic staining after neutral paraformaldehyde fixation, were analyzed by cell fractionation and immunoblotting for BRCA1 (Fig. 3). In all three of these lines, BRCA1 was concentrated in the nuclear fraction. The validity of the fractionation procedure was confirmed by assaying for p300 [a nuclear protein (*3*)], β-tubulin, and GDI-1 (cytosolic proteins). Moreover, there was minimal cross-contamination of nuclear and cytosolic fractions (Fig. 3). Hence, BRCA1 behaved as a nuclear protein in two different analytic tests, one performed with multiple, specific antibodies on cell lines derived from human breast and ovarian cancers. This conclusion differs from that drawn by Chen *et al.* (*1*), who concluded that, in such cell lines (some of which were also tested here) BRCA1 was cytoplasmic, being specifically excluded from the nucleus (*1*).

We did detect a weak cytoplasmic signal with some of our BRCA1 mAbs in certain breast and ovarian carcinoma cell lines. In HMECs, but not in other cell lines, the affinity-purified polyclonal antiserum also gave a cytoplasmic signal. However, two-color immunofluorescence and confocal microscopic analysis did not reveal co-localization of any two cytoplasmic staining signals generated with different monospecific antibodies, which suggests that the cytoplasmic signals represent nonspecific cross-reactions.

An effort was also made to determine the subcellular localization of BRCA1 in tumor cells in alcoholic formalin-fixed, paraffin-embedded sections of primary invasive ductal breast carcinoma (*4*). A similar analysis was performed by Chen *et al.* (*1*). With the use of either of two different BRCA1 mAbs, a variety of different tumor cell staining patterns was noted in the 14 samples we analyzed. They ranged from predominantly nuclear to mainly cytoplasmic to both nuclear and cytoplasmic. By contrast, microwave heating of slides from the same tumor samples, performed with the intention of maximizing antibody access to the available BRCA1 epitope or epitopes before immunostaining, produced a predominantly cytoplasmic signal in 14 out of 14 samples. The discrepancy in the signal observed with and without microwave treatment raised a question as to which, if any, of the detected signals most accurately reflected the true intracellular distribution of BRCA1 in these tumors.

To pursue this question further, we again analyzed aliquots of MCF7 and SKOV-3 cells, where BRCA1 was revealed to be nuclear by two independent criteria. Each cell line was pelleted and divided into two aliquots. One aliquot of each line was fixed in alcoholic formalin and the other in neutral buffered formalin. Cut sections of each pellet were processed with or without microwave treatment in the same manner as the above-noted tumor tissue. One of the two BRCA1 mAbs used in our earlier experiment on sections of primary invasive ductal breast carcinoma was used for immunoperoxidase staining of MCF7 cells (Fig. 4). Identical results were obtained when SKOV-3 cells were reacted with this antibody and when each of the two cells lines, fixed in the same manner, was reacted with the second mAb used in the earlier experiment.

In alcoholic formalin-fixed cells, a strong cytoplasmic staining pattern was seen in all cases. By contrast, in cells fixed in neutral buffered formalin (different from neutral paraformaldehyde) and exposed to microwave heating before immunoperoxidase staining, the BRCA1 signal was predominantly nuclear. However, in neutral buffered formalin-fixed cells that had not undergone microwave treatment, the signal was strongly and exclusively cytoplasmic (Fig. 4).

Thus, cells known to contain exclusively nuclear BRCA1 (as shown by biochemical extraction and by immunostaining performed under certain conditions of fixation) revealed non-nuclear staining under other fixation conditions—those commonly used to analyze tumor sections. This result, along with the observation that differences in BRCA1 staining patterns of breast cancer sections can be linked to variation in fixation or staining conditions, raises questions about the biological significance of detecting largely cytoplasmic BRCA1 staining in any breast cancer section (*1*). Taken together, the results reported here do not support the hypothesis (*1*) that wild-type BRCA1 is specifically excluded from the nucleus in sporadic breast and ovarian cancer.

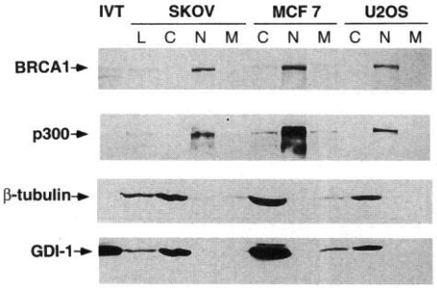

Fig. 3. Full-length BRCA1 protein is nuclear. Cell lines SKOV-3 (ovarian cancer), MCF7 (breast cancer), and U2OS (osteosarcoma) were biochemically fractionated with the use of a method adapted from Lee *et al.* (*6*). For a given cell line, equal amounts of total protein were loaded in each lane. Proteins were separated by 6% SDS-PAGE and probed for BRCA1 with an immunoblot (*5*). IVT, 15 μl of in vitro–translated BRCA1; L, whole-cell lysate; C, cytosolic fraction; N, nuclear fraction; M, membrane fraction. Controls for the quality of fractionation were as follows: p300 (detected with mouse mAb RW128, a nuclear protein (*3*); β-tubulin (antibody is from Boehringer-Mannheim); and GDI-1 (detected with an affinity-purified rabbit polyclonal antiserum). In the experiment shown, there was some widening of lanes in the lower molecular weight regions of the gel.

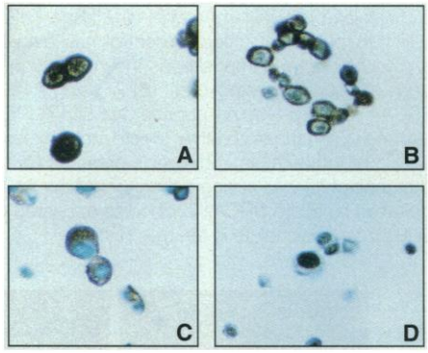

Fig. 4. BRCA1 immunostaining in formalin-fixed, paraffin-embedded pellets of cancer cell lines. Cell lines SKOV-3 (ovarian cancer) and MCF7 (breast cancer) were grown and pelleted. Pellets were divided into two aliquots that were fixed in alcoholic formalin (AF) and neutral buffered formalin (NBF), respectively. Subsequent processing and immunoperoxidase staining methods were identical to those used on sections of primary invasive ductal breast carcinoma (*4*). For a given fixation-microwave combination, similar staining patterns were obtained in sections of pelleted MCF7 or pelleted SKOV-3 cells, with the use of mAb SG11 or mAb MS13 (*2*). The combination shown in this figure—SG11 staining of MCF7 cell pellet sections—is therefore typical of the other cell line–antibody combinations. (**A**) AF fixation, no microwave treatment before staining; (**B**) AF fixation, with microwave treatment before staining; (**C**) NBF fixation, no microwave treatment before staining; and (**D**) NBF fixation, with microwave treatment before staining.

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2. Y. Miki *et al.*, *ibid.* **266**, 66 (1994). We obtained the cDNA for BRCA1 by probing a 293–cell cDNA library. Two partially overlapping fragments were reassembled to produce full-length BRCA1 cDNA, which was then sequenced and found to contain no mutations. Antibodies were raised in mice against a BRCA1-GST fusion protein that contained residues 1 to 304 of BRCA1 (amino terminal segment, MS series of mAbs) or residues 1313 to 1863 of BRCA1 (carboxyl-terminal segment, AP series of mAbs). The SG series of mAbs was raised against the BRCA1 carboxyl-terminal peptide CQELDTYLIPQIPHSHY. Monoclonal antibody fusions were screened by immunoprecipitation of ^{35}S-methionine–labeled, in vitro–translated BRCA1, followed by 6% SDS-PAGE and autoradiography. Positive wells were recloned and rescreened twice, the second subcloning being at limiting dilution, which allowed single-cell clones to be picked.
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4. Alcoholic formalin is composed of 10% buffered formalin (Anatech Ltd, Battle Creek, MI) diluted into 70% ethanol. Immunoperoxidase staining was performed using a Ventana Automated Immunostainer (Ventana Medical Systems, Tucson, AZ). Monoclonal antibody SG11 was used at 1:10 dilution; mAb MS13 was used without dilution. Where microwave heating was used for antigen retrieval, sections were heated in the microwave in citrate buffer pH 6 for 10 minutes, with buffer replacement in between the heating periods. 3-3′ diaminobenzadine was used as the chromogen, and the sample was lightly counterstained with methyl green.
5. Cells were lysed in RIPA buffer (50 mM tris, pH 8.0, 150 mM sodium chloride, 0.1% SDS, 0.1% sodium deoxycholate, 1% NP 40) in the presence of phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate) and protease inhibitors [aprotinin (20 μg/ml), leupeptin (10 μg/ml), phenylmethylsulphonylfluoride (100 μg/ml)], at 4°C. After separation by 6% SDS-PAGE, proteins were transferred to PVDF (Immobilon P, Millipore, Bedford, MA) by electrophoresis in Towbin buffer (25 mM tris, 192 mM glycine, pH 8.3, 0.01% SDS, 0% methanol). The membrane was blocked in either 4% bovine serum albumin (BSA) or 5% nonfat milk in tris-buffered saline (TBS) (0.9% sodium chloride, 20 mM tris, pH 7.4) supplemented with 0.1% sodium azide and 0.05% Tween 20, for 1 hour at room temperature. The primary antibody was mAb MS110 (*2*) at 1:10 dilution of tissue-culture supernatant in TBS-Tween 20 0.05% (TBS-T), with 2% BSA or nonfat milk and 0.1% sodium azide. Washes were in TBS-T. The secondary antibody was affinity-purified goat antibody to mIgG-peroxidase (Boehringer-Mannheim, Indianapolis, IN). The signal was developed using ECL (Amersham, Arlington Heights, IL).
6. W.-H. Lee *et al.*, *Nature* **329**, 642 (1987). Cells were not labeled prior to fractionation. The cytosolic fraction was concentrated using a regenerated cellulose 10,000 NMWL filter unit (ultrafree-MC, Millipore) and equilibrated with lysis buffer. The protein concentration of each fraction was measured with reference to a standard curve, using a detergent-compatible protein assay (Bio-Rad, Hercules, CA). Conditions for the protein immunoblot are given above (*5*).
7. We thank J. Zhou and J. Prioleau for technical assistance. R.S. was supported by a grant from the Dana-Farber Women's Cancer Program. Mouse mAb GG22 (Fig. 1) was a gift from D. Ginsberg; mouse mAb RW128 (Fig. 3) was a gift from R. Eckner; and rabbit polyclonal antiserum to GDI-1 (Fig. 3) was a gift from P. E. Bickel. This work was also funded by grants from the U.S. National Institutes of Health.

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Response: Scully *et al.* state that they find BRCA1 protein exclusively in the nucleus of many types of human cells, including cells derived from breast and ovarian cancer cell lines. Using different reagents (*1*), we also found that BRCA1 was localized in the nucleus in many types of human cells. However, we also found that BRCA1 was localized almost exclusively in the cell cytoplasm of breast and ovarian cancer cell lines. We agree with Scully *et al.* that accurate localization of gene products by immunocytochemistry depends on antibody specificity, as well as on methods of fixation and staining. Let us consider in more detail some of the similarities and differences between the studies.

It is important to determine whether the different antibodies used by each group are specific for BRCA1 alone, or whether they also recognize cross-reacting proteins that may profoundly influence the results of immunohistochemistry (IHC) and subcellular fractionation experiments. In our report, we used two different mouse polyclonal antibodies, raised against large and distinct regions encoded by *BRCA1* exon 11, to characterize the BRCA1 protein.

One of our antibodies was raised against a GST-BRCA1 fusion protein corresponding to amino acids 762 through 1315, and the other, against a GST fusion protein corresponding to amino acids 341 through 758. Both antibodies were purified by preabsorption with GST beads. Both gave essentially identical results, but only one (antibody to BRCA1 762–1315) was emphasized in our report (*1*) because of space limitations. We carefully determined the specificity of these antibodies by immunoprecipitating ^{35}S-labeled BRCA1 and reprecipitating it with either of the two polyclonal antibodies, to minimize contamination with cross-reacting and co-precipitating proteins. Likewise, in protein immunoblots, performed after we immunoprecipitated the protein from cellular lysates and probed with the same antibody, only a single protein migrating at 220 kD was visualized on a full-length blot (Fig. 1A, lanes 1 through 4). These data strongly suggest that our antibodies are specific for BRCA1, possess little if any cross-reactivity, and are appropriate for IHC studies.

We, like Scully *et al.*, have made other polyclonal and monoclonal antibodies against various regions of the 220-kD BRCA1 protein. Most of our antibodies cross-react with other proteins, which suggests that truly specific reagents are difficult to obtain. With the use of our relatively nonspecific antibodies in immunoprecipitation or in straight protein immunoblots of cell lysates, cross-reacting proteins are often much more abundant (by a factor of ten) than is the 220-kD BRCA1 protein itself (Fig. 1, lanes 5 through 8). Furthermore, the cross-reacting antibodies usually show predominant nuclear immunostaining of the same breast cancer cell lines that also demonstrate predominantly cytoplasmic staining with our BRCA1-specific antibodies. Our only truly specific antibodies were purified from polyclonal sera from samples taken soon after immunization of the mice. This result suggests that repeated boosting may favor the more abundant cross-reacting substrates, again making it difficult to obtain specific antibodies indefinitely.

In an attempt to resolve the problems associated with potential antibody cross-reactivity, we have recently created a tagged BRCA1 expression vector. BRCA1 protein is expressed from a plasmid (based on Invitrogen's pCEP4, San Diego, California) that contains the entire 5.5-kb coding sequence of the *BRCA1* cDNA, in-frame with a Flag epitope-tag sequence at the N-terminal region. The tag permits detection of exogenous BRCA1 with the use of the specific antibody to Flag (M2, Kodak, Rochester, New York) (*2*) after the CEP4-BRCA1 construct is transfected into

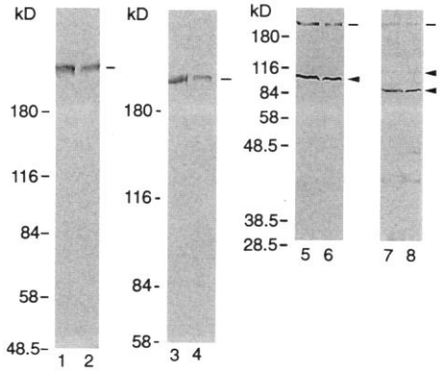

Fig. 1. Protein immunoblot analysis with mouse antibodies to BRCA1. Immunoprecipitation of lysates from three cell lines, HBL100 (lanes 1 and 3), T24 (transitional cell carcinoma of the bladder, lane 2), and T47D breast adenocarcinoma cells (lane 4), each with 2 μl of preabsorbed, polyclonal, antiserum to BRCA1 (amino acids 762 through 1315). Immunoprecipitates were separated by SDS-PAGE. The blot was developed by probing with the same antibody. Lanes 5 through 6: straight Western blotting of HBL100 cell lysates (5 × 10^6 cells, lane 6), using mAb 6B4; the thin horizontal line marks BRCA1, which migrates at about 220 kD, and the arrowhead marks an abundant, cross-reacting protein migrating at about 110 kD. Lanes 7 through 8: same experiment using mAb 24G11; two cross-reacting proteins are marked by arrowheads.

cells. In our recent transfection experiments, Flag-tagged, wild-type BRCA1 was expressed as a full-length protein migrating at about 220 kD. The eight–amino acid Flag epitope did not appreciably alter the mobility of tagged BRCA1 as compared with the untagged protein. In indirect immunofluorescence experiments, tagged BRCA1 localized in the nucleus of transfected, "normal" HBL100 human breast epithelial cells and fibroblasts but remained in the cytoplasm of several breast cancer cell lines, including T47D and MD468 (Fig. 2B). These results support our hypothesis that the BRCA1 protein is mislocated in the cytoplasm of advanced breast cancer cells. They also strongly suggest that exclusion of BRCA1 from the nucleus is a result of defective BRCA1 transportation, rather than of mutations in BRCA1 itself or of artifacts associated with antibody cross-reactivity.

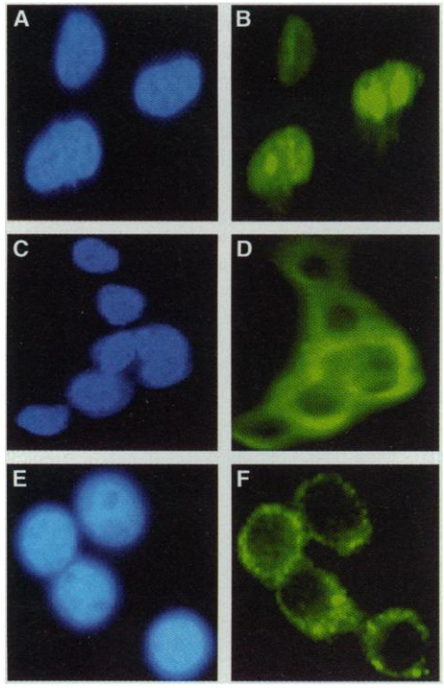

Fig. 2. Studies with epitope-tagged human BRCA1 (immunostaining of cells transfected with Flag-BRCA1). Sixty hours after transfection the cells were fixed with neutral formaldehyde and 0.1% Triton-X, then stained with DAPI as a positive control for nuclear staining (blue fluorescence, **A**, **C**, and **E**). The same cells were also stained for exogenous BRCA1, using the mAb to Flag, M2, as the primary antibody, and with FITC-conjugated sheep antiserum to mouse for detection (**B**, **D**, and **F**). In HBL100 cells, tagged, exogenous BRCA1 localized in the nucleus (A and B), as did the endogenous protein. In breast cancer cell lines T47D (C and D) and MD468 (E and F), staining for BRCA1 remained cytoplasmic.

Scully *et al.* obtained subcellular localization results that were different from ours. As they suggest, these variations may be a result of differences in antibody specificities or immunostaining procedures (fixation and so forth). We have carefully characterized our antibodies (as described above) and believe that they are indeed specific for BRCA1.

Scully *et al.* observed that the apparent localization of BRCA1 by immunostaining with their antibodies was greatly altered by changing fixation methods. We agree that fixation and staining procedures are important to control. All antigens (and therefore their reactivities with specific antibodies) are influenced (masked or unmasked) in varying ways by different types of fixation. The best one can do to study a biological phenomenon with IHC is to standardize these conditions within a particular study. For these reasons, we used the same methodology throughout our experiments, and we fixed cells and tissue sections with neutral buffered formalin. Differences observed within this standardized setting can safely be attributed to differences in biology rather than in technology. Furthermore, in our IHC studies of clinical breast cancers, we always observed a nuclear BRCA1 signal in normal cells accompanying the tumor, but only observed variations in nuclear versus cytoplasmic localization within the tumor cells. We also used, in our IHC studies, a heat-induced antigen retrieval method that included citrate buffer at pH 6.0 and a pressure cooker; this method is similar to the microwave method used by Scully *et al.*, but it is more efficient. We have already mentioned how cross-reacting antibodies can profoundly influence IHC results, entirely independent of fixation and staining procedures.

Scully *et al.* also obtained biochemical fractionation results that were different from ours. With the fractionation method we used in a previous study (3), we found that the 220-kD BRCA1 protein separated with the crude nuclear fraction. However, this fraction was contaminated with significant amounts of cytoplasm and cytoplasmic organelles adhering to the nuclear membrane. When we fractionated cells with a more stringent protocol as described by Eisenman (4), which employed a nuclear wash, the vast majority of BRCA1 protein in T47D breast cancer cells separated with the wash, indicating an extranuclear location. On close inspection, these stringent fractionation results were also consistent with our immunostaining results, in which immunostained BRCA1 had a cytoplasmic, perinuclear distribution (Fig. 2). In collecting data for our report, we did not use MCF7 cells for fractionation studies because these cells were heterogeneous. Two phenotypically distinct population were identified: smaller, anisocytotic cells with both nuclear and cytoplasmic staining for BRCA1; and more uniformly sized cells in which staining for BRCA1 was exclusively cytoplasmic.

The comment by Scully *et al.* and our work appear to agree on one matter: BRCA1 is a 220-kD protein that localizes in the nuclei of normal breast epithelial cells. A recent report, however, identified BRCA1 as a 190-kD secreted protein localizing to membrane vesicles (5). The rabbit polyclonal antibodies used in this latter study were raised against peptides (19 or 20 of the carboxyl-terminal amino acids) identical or nearly identical to those used to generate polyclonal A19 and several of the mAbs used by Scully *et al.* These discrepancies with antibodies that recognize nearly identical epitopes in BRCA1 are difficult to explain. Perhaps antibodies to small epitopes in other, more abundant cellular proteins overlap with those in the carboxyl-terminal of BRCA1. This potential problem raises more questions about the specificity and suitability of such antibodies for immunostaining.

In summary, our previously reported results (*1*) and our new results obtained with Flag-tagged BRCA1 protein show that BRCA1 is aberrantly located in the cytoplasm of many breast cancer cells.

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