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 (CQELDTLYLPQIPHSHY) 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 [35S]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
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 U2OS), 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 whether, 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 cell 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-microscope combination, similar staining patterns were obtained in sections of pelleted MCF7 or pelleted SKOV-3 cells, with the use of mAbs SG11 or mAb MS13 (2). The combination shown in this figure—SG11 staining of MCF7 cell pellet sections—was 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.
1. Y. Chen et al., Science 270, 789 (1995).
2. Y. Miki et al., Ibid. 266, 66 (1994). We obtained the cDNA for BRCA1 by probing a 293-cell cDNA library. One large cDNA fragment was re-assembled 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 (carboxy-terminal segment, AP series of mAbs). The SG series of mAbs was raised against the BRCA1 carboxy-terminal peptide COQELDITYLPQiPShY..

Monoclonal antibody fusions were screened by immunoprecipitation of 35S-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.

3. R. Eckner et al., Genes Dev. 8, 869 (1994).
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 5G11 was used at 1:10 dilution; mAb MS15 was used neat. To distinguish where microwave heating was used for antigen retrieval, sections were heated in the microwave in citrate buffer pH 5-6 for 10 minutes, with buffer replacement in between the heating periods. 3-3’-diaminobenzidine 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, 0.1% sodium orthovanadate) and 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), phenylmethanesulfonyl fluoride (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, pH 8.3, 192 mM glycine, 0.1% SDS, 0.05% 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 MS151 (20) at 1:10 dilution of tissue-culture supernatant in TBS-Tween 20 0.05% (pH 7.4). The secondary antibody was anti-mouse immunoglobulin G-peroxidase (Boehringer Mannheim, Indianapolis, IN). The signal was detected using ECL (Amersham, Arlington Heights, IL).

6. W.-H. Lee et al., Nature 329, 642 (1987). Cells were not labeled prior to fractionation. The cytotoxic fraction was obtained by scraping the regenerating cell layer 10,000 NWML filter unit (ultrafree-MC, Millipore) and equilibrated with lysis buffer. The protein concentration of each fraction was measured with reference to a standard curve of a detergent-complement protein assay (Bio-Rad, Hercules, CA). Conditions for the protein immunoblot are given above (5).

7. We thank J. Zhou and J. Pilch in technical assistance. R.S. was supported by a grant from the Dana-Farber Women’s Cancer Program. Mouse mAb G22 (Fig. 1) was a gift from D. Ginsburg; 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.

16 January 1996; accepted 20 February 1996

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 35S-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 and staining 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 8: straight Western blotting of HBL100 cell lysates (5 × 106 cells, lane 6), using mAb 684; 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.