Aberrant Subcellular Localization of BRCA1 in Breast Cancer

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The *BRCA1* gene product was identified as a 220-kilodalton nuclear phosphoprotein in normal cells, including breast ductal epithelial cells, and in 18 of 20 tumor cell lines derived from tissues other than breast and ovary. In 16 of 17 breast and ovarian cancer lines and 17 of 17 samples of cells obtained from malignant effusions, however, BRCA1 localized mainly in cytoplasm. Absence of BRCA1 or aberrant subcellular location was also observed to a variable extent in histological sections of many breast cancer biopsies. These findings suggest that BRCA1 abnormalities may be involved in the pathogenesis of many breast cancers, sporadic as well as familial.

The cloning of the familial breast and ovarian cancer gene *BRCA1* (*1*) was a significant milestone in breast cancer research. Nonetheless, although *BRCA1* has been linked to greater than 45% of site-specific, inherited breast cancers and 80% of families with breast and ovarian cancer (*2*), no sporadic breast cancers and only about 10% of sporadic ovarian cancers have been found to harbor *BRCA1* mutations (*1*, *3*). Thus the general function of BRCA1 in the pathogenesis of sporadic breast cancers, which account for about 95% of such neoplasms (*4*), has been unproven to date (*5*).

BRCA1 complementary DNA encodes a 1863–amino acid protein whose predicted structure includes two zinc finger domains near the NH_2-terminus and an acidic COOH-terminal domain, leading to speculation that the BRCA1 protein is a transcription factor (*1*, 6). To help characterize BRCA1, we generated polyclonal antibodies to BRCA1 (anti-BRCA1) by creating a glutathione-S-transferase (GST)–BRCA1 fusion protein containing amino acids encoded by a 3′ portion of *BRCA1* exon 11 (*7*). Anti-BRCA1 serum specifically immunoprecipitated a protein with a molecular mass of 220 kD in HBL100 human diploid breast epithelial cells metabolically labeled with ^{35}S-methionine (Fig. 1A). The protein migrated at approximately the size predicted from the 1863–amino acid sequence (*1*). Because anti-BRCA1 serum coprecipitated at least five proteins other than BRCA1, a double immunoprecipitation involving denaturation was performed (*8*) and detected only the 220-kD protein (Fig. 1A, lane 3). Two additional polyclonal antibodies were used in similar experiments. C20, directed against an epitope near the COOH-terminus (*9*), and BRCA1-Bgl, raised against a fusion protein with sequences encoded by the more 5′ portion of exon 11 (*7*), identified the same protein as the first antibody [Fig. 1A, lane 6 and (*10*)]. The same results were obtained when each step in the double immunoprecipitation was performed with a different polyclonal antibody. These immunological results demonstrated that the 220-kD protein is the *BRCA1* gene product. The immunoprecipitate of lysate from HBL100 cells labeled with [^{32}P]phosphoric acid contained only a single, more slowly migrating species (lane 7) and thus showed that BRCA1 is a phosphoprotein.

BRCA1 is present not only in normal breast epithelial cells like the HBL100 line, but in all breast cancer lines tested (Fig. 1B). It appears to be expressed largely intact in these cells, because the proteins identified by ^{32}P labeling and immunoprecipitation with anti-BRCA1 all migrated in the gel at ~220 kD. Thus BRCA1 is not mutated by truncation in most breast cancer cell lines. In tumor lines derived from tissues other than breast, BRCA1 appears to be more abundant than in breast cancer lines; it can be detected more easily in bladder, cervical, colon, and other cancers by labeling with ^{35}S-methionine (Fig. 1C).

To determine the subcellular localization of BRCA1, we fractionated HBL100 cells into nuclear, cytoplasmic, and membrane components (*11*). BRCA1 was detected in normal cells mainly in nuclei (Fig. 2A). Furthermore, indirect immu-

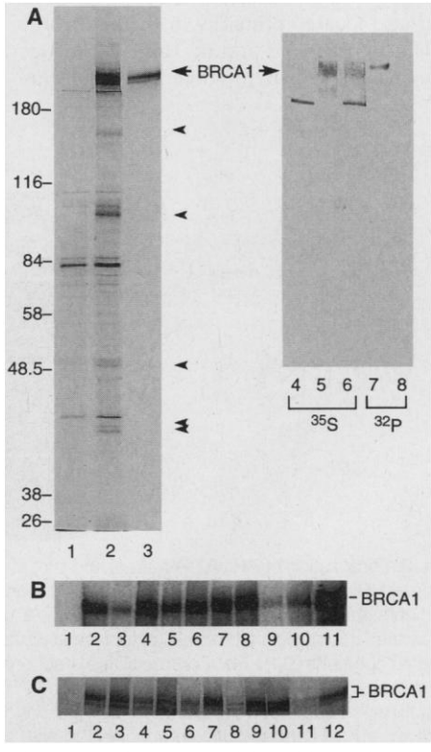

Fig. 1. (**A**) Identification of BRCA1. Diploid human breast epithelial cells (HBL100, about 1 × 10^7 cells per lane) were incubated with ^{35}S-methionine (lanes 1 to 6) [^{32}P]phosphoric acid (lanes 7 and 8). Proteins from lysates were then immunoprecipitated by excess preimmune mouse serum (lanes 1, 4, and 8) or by mouse polyclonal anti-BRCA1 (lane 2), separated by SDS–polyacrylamide gel electrophoresis, and autoradiographed. Arrowheads indicate proteins coimmunoprecipitated by anti-BRCA1 serum. Immunoprecipitated proteins were dissociated from anti-BRCA1 and immunoprecipitated again with an excess of the same antibody to visualize only BRCA1 (lane 3). The same protein was immunoprecipitated by two different antibodies, anti-BRCA1 (lane 5) and C20 (lane 6). One protein species labeled with [^{32}P]phosphate was also immunoprecipitated by anti-BRCA1 (lane 7) but not by preimmune serum (lane 8). (**B**) Detection of full-length BRCA1 in normal breast epithelial cells and breast cancer cell lines (*17*). Human breast cell lines (5 × 10^6 cells per lane) were labeled with [^{32}P]phosphoric acid. Lane 1, HBL100 lysate immunoprecipitated by preimmune mouse serum. Cell lysates in lanes 2 to 11 immunoprecipitated by anti-BRCA1: lane 2, T47D; lane 3, MCF7; lane 4, MB468; lane 5, MB175-7; lane 6, MB-361; lane 7 MB-231; lane 8, MB-435S; lane 9, MB415; lane 10, HS578T; and lane 11, HBL100 (*18*). (**C**) Full-length BRCA1 is expressed in tumor cell lines derived from tissues other than breast. Human cell lines (~2 × 10^6 per lane) were metabolically labeled with ^{35}S-methionine. One lysate was immunoprecipitated by preimmune serum (lane 1) and all others by anti-BRCA1 (lanes 2 to 12). Cell lines: lanes 1 and 2, T24 [transitional cell carcinoma (TCC) of the bladder]; lane 3, 5637 (TCC bladder); lane 4, DU145 (prostate carcinoma); lane 5, CAOV3 (ovarian carcinoma); lane 6, RD (rhabdomyosarcoma); lane 7, HCT116 (colon carcinoma); lane 8, SW620 (colon carcinoma); lane 9, C4II (cervical carcinoma); lane 10, MS751 (cervical carcinoma); lane 11, SAOS-2 (osteosarcoma); and lane 12, U2OS (osteosarcoma).

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nostaining of intact cells, including HBL100, several other normal cell lines, and tumor cells derived from tissues other than breast or ovary also localized BRCA1 to nuclei (Fig. 2, B and C; Table 1). In contrast, BRCA1 was detected mainly in the cytoplasm of almost all breast cancer cell lines tested (Fig. 2C; Table 1). In 14 of 17 cell lines established from breast cancers and in 2 of 3 lines from ovarian cancers, BRCA1 staining was principally cytoplasmic. For two other breast cancer lines, both nuclear and cytoplasmic staining was observed in the same cells. One line, MDA-MB361, which was originally derived from a brain metastasis (*12*), contained two distinct populations of cells: a less abundant fraction of larger, more heterogeneous cells in which BRCA1 localized to the nuclei, and a more abundant fraction of smaller, more homogeneous cells in which BRCA1 localized to the cytoplasm. Similar results were obtained by cell fractionation in several of the same cell lines. These results suggest that BRCA1 is located aberrantly in the cytoplasm of most breast and ovarian cancer cell lines.

Next we examined primary cells from malignant pleural effusions and biopsy sections from patients with breast cancer. In all of the primary malignant effusion cells, obtained from 17 different patients, BRCA1 was also located primarily in the cytoplasm (Fig. 2D, panels n and p; Table 1). Other tumor cells grown in suspension (such as leukemia lines CEM, HL60, and Molt4) or metastatic to pleura (K562 and U937) stained mainly in nuclei (Table 1).

Table 1. Subcellular location of BRCA1 in cell lines, primary tumor cells from malignant pleural effusions, and tissue biopsy sections.

Tissue or tumor of origin	Cases (*n*)	BRCA1 location			BRCA1 absent
		Nucleus	Cytoplasm	Both	
Established lines					
Normal fibroblast	2	2	0	0	0
Renal epithelium	1	1	0	0	0
Bladder carcinoma	3	3	0	0	0
Cervical carcinoma	2	2	0	0	0
Leukemia or lymphoma	4	4	0	0	0
Osteosarcoma	2	2	0	0	0
Prostate carcinoma	1	1	0	0	0
Rhabdomyosarcoma	2	2	0	0	0
Breast epithelium	1	1	0	0	0
Breast adenocarcinoma	18	1	15	2	0
Ovarian carcinoma	3	1	2	0	0
Malignant effusions					
Breast adenocarcinoma	17	0	17	0	0
Ovarian carcinoma	8	0	8	0	0
Leukemia or lymphoma	2	2	0	0	0
Fixed tissue sections					
Infiltrating lymphocytes	50	50	0	0	0
Breast carcinoma	50	8	6	34	2

Breast tumor cells in culture and from malignant pleural effusions were all derived from advanced, metastatic cancers. To determine whether BRCA1 also localized aberrantly in primary tumors, we used the same polyclonal anti-BRCA1 serum to stain cells in tissue sections. Complete or partial localization of BRCA1 was shown in the cytoplasm of most breast cancer cells (Fig. 3). In 50 biopsies, BRCA1 staining was mainly cytoplasmic in 6 (12%), cytoplasmic and nuclear to a variable extent in 34 (68%), primarily nuclear in 10 (20%), and absent in 2 (4%) (Table 1). These results demonstrate abnormal subcellular localization of BRCA1 in primary breast tumors as well as those that are distantly metastatic. Complete misloca-

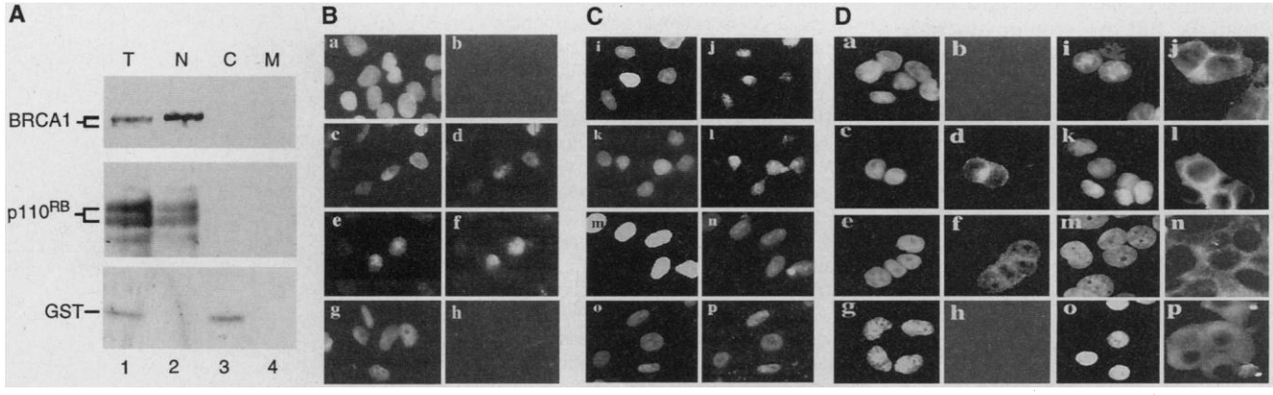

Fig. 2. Localization of BRCA1 in normal and breast cancer cells. (**A**) Fractionation of HBL100 cells. Cells (1.5×10^7) were labeled with ^{35}S-methionine; 5×10^6 cells were left unfractionated (total or T, lane 1) and the remainder were separated into nuclear (N, lane 2), cytoplasmic (C, lane 3), and membrane (M, lane 4) fractions (*11*). For control of the fractionation procedure, p110^{RB} served as a marker for nuclear distribution and GST for cytoplasmic distribution. Small aliquots of unfractionated (5×10^4) and fractionated cells (1.5×10^5) were lysed and directly immunoblotted with a monoclonal antibody to p110^{RB}. Similar aliquots were incubated with GST beads, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue to visualize the expected 26-kD glutathione-S-transferase (GST) band. (**B**) Detection of BRCA1 in the nuclei of intact HBL100 cells by indirect immunofluorescence staining. (a, c, e, g) DAPI staining to mark nuclei; (b, d, f, h) immunofluorescence staining of the same cells (*19*). (a and b) Preimmune serum as primary antibody; (c and d) anti-BRCA1 as primary antibody; (e and f) anti-BRCA1 preabsorbed with GST antigen; (g and h) anti-BRCA1 preabsorbed with the GST-BRCA1 fusion protein. (**C**) Detection of BRCA1 in the nuclei of cell lines derived from tissues other than breast. (i, k, m, o) DAPI staining; (j, l, n, p) BRCA1 staining. (i and j) DU145 (prostate carcinoma) cells; (k and l) Rat2 fibroblasts; (m and n) T24 (TCC bladder) cells; (o and p) CV1 (monkey kidney epithelial) cells. (**D**) Cytoplasmic localization of BRCA1 in breast cancer cells. (a) through (h) breast cancer line T47D; (k and l) breast cancer line MCF7; (m and n) cells from primary malignant effusion #22550; (o and p) cells from primary effusion #23159. (a, c, e, g, i, k, m, o) DAPI staining; (b) preimmune serum as primary antibody; (d) polyclonal anti-BRCA1 primary antiserum; (f) anti-BRCA1 preabsorbed with GST; (h) anti-BRCA1 preabsorbed with GST-BRCA1 fusion protein; (i through p) anti-BRCA1 primary antibody, preabsorbed with glutathione-S-transferase. Magnification is the same in (B) through (D).

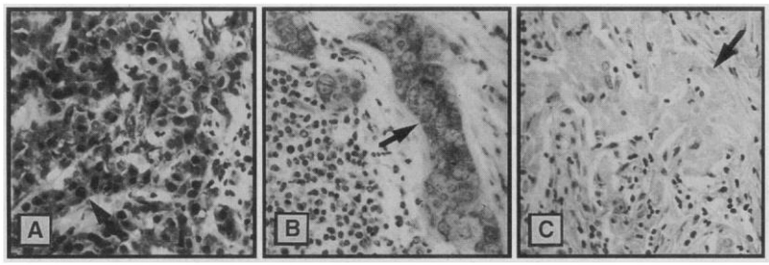

Fig. 3. Primary breast cancer sections stained for BRCA1 by the immunoperoxidase method (*18*). (**A**) BRCA1 localized to both cytoplasm and nuclei; (**B**) BRCA1 localized only to cytoplasm; (**C**) BRCA1 staining absent. The small, round, dark signals in all sections are lymphocyte and stromal cell nuclei. Original magnification, ×400.

tion of BRCA1 appears to be more common in end-stage breast cancer, but nonetheless occurs to a variable extent in the great majority of tumors in a random survey. The 4% of tumors that lack BRCA1 altogether may represent familial cases; such a percentage corresponds well with the similar, small incidence of BRCA1 mutations in breast cancers of all kinds (*4*). Note that in the stromal cells and lymphocytes from the tumor in Fig. 3C, staining for BRCA1 is nuclear, whereas breast tumor cells in the same sections fail to stain at all with the same procedure.

The subcellular mislocation of BRCA1 protein suggests that abnormalities in BRCA1 are fundamental to the genesis or progression of most breast cancers. BRCA1 may be inactivated by intragenic mutation, as an early event, in hereditary breast cancers, whereas in most nonhereditary breast cancers it may be inactivated indirectly by mislocation in cytoplasm. This explanation would be consistent with the earlier age at onset and more rapid progression of familial forms of breast cancer as compared with sporadic forms (*3*). It would also account for the apparent greater incidence of complete BRCA1 mislocation in metastatic tumor cells.

The molecular mechanism by which BRCA1 is mislocated to the cytoplasm in breast cancer cells awaits further investigation. The BRCA1 amino acid sequence does not have typical bipartite nuclear localization signals (NLSs) (*1*), but does contain at least two other putative NLSs (*13*). These signals (NKLKRKRRP, amino acids 419 to 427; and NRLRRKS, amino acids 609 to 615) are similar to sequences found in estrogen, progesterone, and other steroid hormone receptor molecules (*13*, *14*). To be activated and to redistribute from a primarily cytoplasmic location to the nucleus, steroid hormone receptors require binding to their ligands, conformational changes, and perhaps dimerization (*15*). BRCA1 may normally localize to the nucleus in a similar manner, by dissociation from proteins that anchor it in the cytoplasm, as a passenger with other nuclear proteins, or after modification to expose its own potential NLS. Similar transport mechanisms have been demonstrated for other transcription factors including SV40 large T antigen and c-Fos (*16*). The mutations of molecules involved in the pathway of BRCA1 transport from its site of synthesis to sites of action in the nucleus may be alternative ways to inactivate the same crucial protein in many sporadic breast cancers.

REFERENCES AND NOTES

1. Y. Miki *et al.*, *Science* **266**, 66 (1994).
2. D. F. Easton *et al.*, *Am. J. Hum. Genet.* **52**, 678 (1993).
3. P. A. Futreal *et al.*, *Science* **266**, 120 (1994); L. S. Friedman *et al.*, *Nat. Genet.* **8**, 399 (1994); D. Shattuck-Eiders *et al.*, *J. Am. Med. Assoc.* **273**, 535 (1995); L. Hosking *et al.*, *Nat. Genet.* **9**, 343 (1995); S. D. Merajver *et al.*, *ibid.*, p. 439.
4. E. B. Claus, W. D. Thompson, N. Risch, *Am. J. Hum. Genet.* **48**, 232 (1991).
5. J. Boyd, *Nat. Genet.* **9**, 335 (1995); L. H. Castilla *et al.*, *ibid.* **8**, 387 (1994).
6. B. Vogelstein and K. W. Kinzler, *Cell* **79**, 1 (1994).
7. In the creation of glutathione-S-transferase (GST)–BRCA1 fusion constructs, the polymerase chain reaction was used to amplify two exon *BRCA1* fragments from WI 38 cell (human diploid lung) genomic DNA. A fragment of ~1.9 kb was amplified with two 27-nucleotide primers synthesized according to the published BRCA1 sequence: BRCA9 [5′-TTGCAAACTGAAAGATCTGTAGAGAGT-3′], upstream of a Bgl II site, and BRCA7 [5′-TTCCAAGCCCGTTCCTCTTTCTTCCAT-3′], downstream of a Bam HI site. The amplified genomic DNA was then digested with Bgl II and Bam HI to create a 1.8-kb fragment from codons 762 to 1315. This fragment was purified and subcloned into the GST expression vector pGEX-2T to create pGST-BRCA1. For creation of a second plasmid, *GST-BRCA1-Bgl*, another 27-nucleotide primer, BRCA8 [5′-GATTTGAACACCACTGAGAAGCGTGCA-3′], beginning at codon 245, and primer BRCA9 were used to amplify a 3.2-kb fragment comprising almost all of exon 11. This fragment was then digested with Bgl II to create a 1.3-kb fragment from codons 341 to 748, which was subcloned into a modified pGEX-2T. Each of the two fusion proteins was expressed in *Escherichia coli* and purified with glutathione-sepharose beads for use as an antigen in mice. Serum from immunized mice was then preabsorbed on GST affinity columns. The serum raised against the first GST-BRCA1 protein was used in all experiments illustrated in the figures. Preimmune serum was obtained from the same mice and used at the same dilution.
8. Immunoprecipitation was performed by labeling HBL100 cells with ^{35}S-methionine, lysing them in lysis-250 buffer, and immunoprecipitating with anti-BRCA1, as previously described for retinoblastoma protein [P.-L. Chen, P. Scully, J.-Y. Shew, J. Y. J. Wang, W.-H. Lee., *Cell* **58**, 1193 (1989)]. Immunoprecipitated proteins were boiled in a denaturing buffer [20 mM tris-HCl (pH 7.4), 50 mM NaCl, 1% SDS, and 5 mM dithiothreitol] for 5 min, diluted 10-fold with a lysis-50 buffer containing different detergents [20 mM tris-HCl (pH 7.4), 50 mM NaCl, 1% NP-40, and 1% deoxycholate], and reimmunoprecipitated by anti-BRCA1 in the same buffer. This doubly immunoprecipitated protein was then washed with lysis-250 buffer before separation by SDS–polyacrylamide gel electrophoresis (SDS-PAGE).
9. Rabbit polyclonal antibody C20, raised against a synthetic peptide corresponding to amino acids 1843 to 1862 of BRCA1, was purchased from Santa Cruz Biotechnology, Inc.
10. Y. Chen *et al.*, data not shown.
11. Y. Chen, P.-L. Chen, W.-H. Lee, *Mol. Cell. Biol.* **14**, 6764 (1994); H. D. Abrams, L. R. Rohrschneider, R. N. Eisenman, *Cell* **29**, 427 (1982).
12. R. Cailleau, M. Olive, Q. V. Cruceger, *In Vitro* **14**, 911 (1978).
13. T. Boulikas, *J. Cell. Biochem.* **55**, 32 (1994).
14. J. L. Arriza *et al.*, *Science* **237**, 268 (1987); M. Danielsen, J. P. Northrop, G. M. Ringold, *EMBO J.* **5**, 2513 (1986); S. Green *et al.*, *Nature* **320**, 134 (1986); P. Kastner *et al.*, *EMBO J.* **9**, 1603 (1990); V. Laudet *et al.*, *Nucleic Acids Res.* **19**, 1105 (1991).
15. B. M. Forman and H. H. Samuels, *Mol. Endocrinol.* **4**, 1293 (1990); E. V. Jensen, *Curr. Top. Pathol.* **83**, 365 (1991).
16. J. Schneider, C. Schindewolf, K. van Zee, E. Fanning, *Cell* **54**, 117 (1988); P. Roux *et al.*, *ibid.* **63**, 341 (1990); T. Moll, G. Tebb, U. Surana, H. Robitsch, K. Nasmyth, *ibid.* **66**, 743 (1991).
17. Established cell lines were obtained from American Type Tissue Collection. Malignant cells from pleural effusions, immediately after being withdrawn from patients, were washed in 50:50 Ham's F-12–Dulbecco's modified Eagle's medium (DMEM) and frozen in liquid nitrogen without passage, in the same medium plus 50% fetal calf serum (FCS) and 10% dimethyl sulfoxide. Before fixation for immunostaining the cells were washed, then plated for 12 hours in Ham's F-12–DMEM plus 10% FCS. Viable cells were cytospun onto glass cover slips where they were fixed as described for established cell lines.
18. Sections 5-μm-thick from randomly selected, formalin-fixed, paraffin-embedded, breast cancer biopsies in our tumor bank were immunostained by a modification of the avidin-biotin-horseradish peroxidase complex (ABC) method [S. M. Hsu, L. Rainer, H. Fanger, *J. Histochem. Cytochem.* **29**, 577 (1981)]. Anti-BRCA1 was used at 1:100 dilution. Both cases of invasive breast cancer showing no cytoplasmic or nuclear immunostaining for BRCA1 did show positive immunostaining for the nuclear proliferation antigen MiB1.
19. Indirect immunofluorescence procedures have been described [T. Durfee, M. A. Mancini, D. Jones, S. J. Elledge, W.-H. Lee, *J. Cell. Biol.* **127**, 609 (1994)]. Briefly, cells grown on cover slips were fixed with 4% formaldehyde and 0.1% Triton-X in phosphate-buffered saline (PBS) and permeabilized with 0.05% Saponin in water. Fixed cells were then blocked with 10% normal goat serum plus 0.5% NP-40 in PBS, incubated with mouse polyclonal anti-BRCA1 primary antiserum (1:1000 dilution), washed, and incubated with fluorescein-tagged goat antibody to mouse immunoglobulin G. At the end of the secondary antibody incubation, one drop of 4,6-diamidino-2-phenolindole propidium iodide (DAPI) was added to the cells for 10 min to stain DNA. Cells were then viewed and photographed under a fluorescence microscope.

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