Elevated expression of protein regulator of cytokinesis 1, involved in the growth of breast cancer cells

Arata Shimo,[1,2] Toshihiko Nishidate,[1] Tomohiko Ohta,[2] Mamoru Fukuda,[2] Yusuke Nakamura[1] and Toyomasa Katagiri[1,3]

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-Ku, Tokyo 108-8639; [2]Division of Breast and Endocrine Surgery, Department of Surgery, St Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan

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To elucidate molecular mechanisms of mammary carcinogenesis and discover novel therapeutic targets for breast cancer, we previously carried out a genome-wide expression profile analysis of 81 breast cancer cases by means of a combination of cDNA microarray and laser microbeam microdissection. Among the upregulated genes, we focused on the functional significance of protein regulator of cytokinesis 1 (*PRC1*) in the development of breast cancer. Western blot analysis using breast cancer cell lines revealed a significant increase in endogenous PRC1 levels in G_2/M phase. Treatment of breast cancer cells with small interfering RNA against PRC1 effectively suppressed its expression and inhibited the growth of breast cancer cell lines T47D and HBC5. Furthermore, we found an interaction between PRC1 and kinesin family member 2C/mitotic centromere-associated kinesin (KIF2C/MCAK) by coimmunoprecipitation and immunoblotting using COS-7 cells, in which these molecules were introduced exogenously. These findings suggest the involvement of a PRC1–KIF2C/MCAK complex in breast tumorigenesis, and this complex should be a promising target for the development of novel treatments for breast cancer. (*Cancer Sci* 2007; 98: 174–181)

Breast cancer is a complex disease characterized by the accumulation of genetic and epigenetic changes in a large number of genes.[1] The multistep model of mammary carcinogenesis, that is, transformation from normal cells to atypical ductal hyperplasia, through to ductal carcinoma *in situ*, and then invasive ductal carcinoma, is the same as that of cancers in other tissues. Obviously, as molecular factors leading to the development of primary breast cancer, its progression, and its metastasis are not fully clarified, their better understanding would lead to identification of target molecules for the development of more effective tools for prevention and treatment of this disease.

Gene expression profiles obtained by cDNA microarray analysis can provide a considerable amount of information for characterizing the nature of individual cancers.[2,3] Through genome-wide expression profile analysis, we have isolated a number of oncogenes that are involved in the development and progression of hepatocellular carcinoma,[4,5] pancreatic cancer,[6,7] prostate cancer,[8,9] synovial sarcoma[10,11] and renal cell carcinoma.[12] Such molecules are considered to be good candidate molecules for the development of new therapeutic modalities.

Because cytotoxic drugs often cause severe adverse reactions, thoughtful selection of novel target molecules on the basis of well-characterized mechanisms of action should be very helpful to develop effective anticancer drugs with minimal risk of adverse reactions. Toward this goal, we carried out expression profile analysis of 81 breast tumors and 29 normal human tissues by means of a cDNA microarray representing 23 040 genes.[13,14] The data from these experiments should provide important information about breast tumorigenesis, and will also be valuable for identifying candidate genes whose products might serve as diagnostic markers or as molecular targets for the treatment of breast cancer.

Here we report significant overexpression of *PRC1* in breast cancer cells and its undetectable level of expression in normal human tissues except testis and thymus. We further demonstrate that downregulation of *PRC1* expression results in growth suppression of breast cancer cells. In addition, we demonstrate the interaction of PRC1 with KIF2C/MCAK. Our findings shown in this paper suggest that PRC1 might play critical roles in tumor cell growth and be a promising target for the development of anticancer drugs to breast cancer.

Materials and Methods

Breast cancer cell lines and clinical samples. Human breast cancer cell lines BT-20, BT-474, MCF-7, SKBR3, HCC1937, MDA-MB-435S, YMB-1, T47D, HBL100 and MDA-MB-231, as well as immortalized human mammary cell line HBL100 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA), and were cultured under their respective depositors' recommendations. HMEC was purchased from Cambrex BioScience (Walkersville, MD, USA). HBC4 and HBC5 cell lines were kindly provided by Dr Yamori of Molecular Pharmacology (Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research). All cells were cultured in appropriate media: RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) for HBC4, HBC5, HCC1937, YMB-1 and T47D (with 2 mM L-glutamine); Dulbecco's modified Eagle's medium (Sigma-Aldrich) for BT-474 and HBL100; EMEM (Sigma-Aldrich) for BT-20 and MCF-7 (with 0.01 mg/mL insulin); McCoy (Sigma-Aldrich) for SKBR3 (with 1.5 mM L-glutamine); L-15 (Roche, Basel, Switzerland) for MDA-MB-435S and MDA-MB-231; and MEGM (Cambrex BioScience) for HMEC. Each medium was supplemented with 10% fetal bovine serum (Cansera International, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). MDA-MB-231 cells were maintained at 37°C in an atmosphere of humidified air without CO_2. The other cell lines were maintained at 37°C in an atmosphere of humidified air with 5% CO_2. Tissue samples from surgically resected breast cancers, and their corresponding clinical information, were obtained from the Department of Breast Surgery

[3]To whom correspondence should be addressed. E-mail: tkatagi@ims.u-tokyo.ac.jp
Abbreviations: EMEM, Eagle's minimum essential medium; PRC1, protein regulator of cytokinesis 1; siRNA, small interfering RNA; β2MG, β2-microgloblin; FACS, fluorescence-activated cell sorting; HA, hemagglutinin; His, histidine; HMEC, human mammalian epithelial cell; KIF2C/MCAK, kinesin family member 2C/mitotic centromere-associated kinesin; MEGM, mammary epithelial growth medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodium dodecylsulfate; V1, variant 1; V2, variant 2; V3, variant 3.

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(Cancer Institute Hospital, Tokyo, Japan) after obtaining written informed consent.

Semiquantitative RT-PCR analysis. We extracted total RNA from each of the microdissected breast cancer clinical samples and normal ducts, breast cancer cell lines and normal human tissues, and then carried out T7-based amplification and RT as described previously.[13] We used the same breast cancer samples for semiquantitative RT-PCR analysis as for cDNA microarray analysis. We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR by monitoring *β2MG* as a quantitative control. The sequences of each set of primers were as follows: 5′-GTGGTCCTAGGAGACTTGGTTTT-3′ and 5′-TACATGCATACCCCCAACAA-3′ for the common sequence among each variant of *PRC1*; 5′-AACGGCAGCATCCTGAGTG-3′ and 5′-ATGTTGGTTGAATTGAGGATT CC-3′ for detection of all variants of *PRC1* (V1, 200 bp; V2, 158 bp; V3, 81 bp); 5′-ACTCTAGGACTTGCATGATTGCC-3′ and 5′-TGGGTGTCA-AACCAAACAGA-3′ for *KIF2C*/*MCAK* and 5′-AACTTAGAG-GTGGGAGCAG-3′ and 5′-CACAACCATGCCTTACTTTATC-3′ for *β2MG*.

Northern blot analysis. Breast cancer northern blot membranes were prepared as described previously.[15] Human multiple-tissue northern blots (Takara Clontech, Kyoto, Japan) were hybridized with [α^{32}P]-dCTP-labeled PCR products of *PRC1* (GenBank accession no. NM_003981) prepared by RT-PCR (see below). Pre-hybridization, hybridization and washing were carried out according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for 14 days. A specific probe (454 bp) for *PRC1* was prepared by RT-PCR using the primer set 5′-GTGGTCCTAGGAGACTTGGTTTT-3′ and 5′-TACATGCATACCCCC AACAA-3′ within the common sequence among the three variants of *PRC1*, and was labeled radioactively using the megaprime DNA labeling system (GE Healthcare, Buckinghamshire, UK).

Construction of expression vectors. To construct the *PRC1V1* and *KIF2C*/*MCAK* expression vectors, the entire coding sequences of the *PRC1V1* (GenBank accession no. NM_003981) and *KIF2C*/*MCAK* (GenBank accession no. NM_006845) cDNAs, respectively, were amplified by PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). The primer sets were as follows: *PRC1*-forward, 5′-CCGGAATTCTCCGCCATGAGGAGAAGTGA-3′ (underline indicates the *Eco*RI site); *PRC1*-reverse, 5′-TTGCCGCTCGAGGGACTGGATGTTGGTTGAA-3′ (underline indicates the *Xho*I site); *KIF2C*/*MCAK*-forward, 5′-CCGGAATTCATGGCCATGGACTCGTCG-3′ (underline indicates the *Eco*RI site); and *KIF2C*/*MCAK*-reverse; 5′-GCTCCGCTCGAGCTGGGGCCGTTTCTT-3′ (underline indicates the *Xho*I site). These PCR products were cloned into the *Eocr*I and *Xho*I sites of the pCAGGS-nHA vector with a C-terminal HA-tag and the pCAGGSn3FC vector with an N-terminal Flag-tag, respectively. All of the constructs were confirmed by DNA sequencing (ABI3700; PE Applied Biosystems, Foster, CA, USA).

Generation of anti-PRC1-specific polyclonal antibody. Plasmids designed to express two fragments of PRC1 (and 234–360 amino acids) with a His-tag at their C-terminus were prepared using pET21 vectors (Novagen, Madison, WI, USA). The two recombinant peptides were expressed in the *Escherichia coli* BL21 codon-plus strain (Stratagene, La Jolla, CA, USA), and purified using Ni-NTA resin agarose (Qiagen, Valencia, CA, USA) according to the supplier's protocols. The purified recombinant proteins were mixed together and then used for immunization of rabbits (Medical and Biological Laboratories, Nagoya, Japan). The immune sera were subsequently purified on antigen affinity columns using Affigel 15 gel (Bio-Rad, Hercules, CA, USA) according to supplier's instructions. Affinity-purified anti-PRC1 antibodies were used for western blot, immunocytochemical and immunohistochemical analyses as described below. We confirmed that this antibody could specifically recognise endogenous PRC1 proteins in MCF-7 breast cancer cells by western blot analysis.

Western blot analysis. To detect the endogenous PRC1 protein in breast cancer cell lines (BT-20, HBC4, HBC5, HCC1937, MCF-7, MDA-MB-231, MDA-MD-435S, SKBR3, T47D, YMB-1, BT474 and HBL100) and HMEC, each of these cells was lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40) with 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). The amount of total protein was estimated using a protein assay kit (Bio-Rad), and the proteins were mixed with SDS sample buffer and boiled for 3 min before loading into a 10% SDS-PAGE gel. After electrophoresis, the proteins were blotted onto nitrocellulose membrane (GE Healthcare). After blocking with 4% BlockAce (Dainippon Pharmaceutical Co., Osaka, Japan) the membranes were incubated with anti-PRC1 polyclonal antibody for detection of endogenous PRC1 proteins. Finally, the membranes were incubated with horseradish peroxidase conjugated-secondary antibody, and bands were visualized using ECL detection reagents (GE Healthcare). β-Actin served as a loading control.

Immunocytochemical staining. To examine the subcellular localization of endogenous PRC1 proteins in breast cancer cell lines HBC5, T47D and MCF-7, we seeded the cells at 1×10^5 cells per well (Laboratory-Tek II chamber slide; Nalgen Nunc International, Naperville, IL, USA). After the 24-h culture, cells were fixed with PBS(–) containing 4% paraformaldehyde for 15 min, and rendered permeable with PBS(–) containing 0.1% Triton X-100 at 4°C for 2.5 min. Subsequently, the cells were covered with 3% bovine serum albumin in PBS(–) at 4°C for 12 h to block non-specific antibody binding, followed by incubation with a rabbit anti-PRC1 polyclonal antibody diluted at 1:1000. After washing with PBS(–), the cells were stained with an Alexa488-conjugated antirabbit secondary antibody (Molecular Probes, Eugene, OR, USA) diluted 1:1000. Nuclei were counterstained with 4′,6′-diamidine-2′-phenylindole dihydrochloride. Fluorescent images were obtained under a TCS SP2 AOBS microscope (Leica, Tokyo, Japan).

Immunohistochemical staining. The expression patterns of PRC1 proteins in breast cancer and normal human tissues were examined as described previously.[12] Slides of paraffin-embedded breast cancer specimens (sample no. 234, 240 and 179), normal mammary tissue (sample no. 10441) and other normal human tissues (lung, heart, liver, kidney and testis) (Biochain, Hayward, CA, USA) were processed for antigen retrieval by autoclaving (108°C, 15 min) in antigen retrieval solution at high pH (DAKO, Cytomaton, Carpinteria, CA, USA), and treated with peroxidase blocking regent (DAKO Cytomaton). Tissue sections were incubated with the anti-PRC1 polyclonal antibody (1:100) followed by horseradish peroxidase-conjugated secondary antibody (DAKO Cytomaton). Antigen was visualized with substrate chromogen (DAKO liquid DAB chromogen; DAKO Cytomaton). Finally, tissue specimens were stained with hematoxylin to discriminate the nucleus from the cytoplasm.

Gene silencing effect of siRNA. We established a vector-based RNA interference expression system using psiU6BX3.0 siRNA expression vectors.[16] The siRNA expression vectors against PRC1 (psiU6BX3.0-PRC1) and a mock control (psiU6BX3.0-Mock) were prepared by cloning double-stranded oligonucleotides into the *Bbs*I site in the psiU6BX3.0 vector. The target sequences of synthetic oligonucleotides for siRNA were as follows: 5′-GGAAAGACTCATCAAAAGC-3′ for Si-#1; 5′-GCATATCCGT-CTGTCAGAA-3′ for Si-#2; 5′-CAGAAAAAGGAGAGAAAAC-3′ for Si-#3; 5′-**T**CATATCC**C**TCTGTCAGA**T**-3′ for Si-m#1; and 5′-**T**CATATCC**C**TCTGT**A**AGA**T**-3′ for Si-m#2 (bold letters indicate mismatched sequence in Si-#2). All of the constructs were confirmed by DNA sequencing.

Human breast cancer cell lines T47D and HBC5 were plated onto 10-cm dishes (1×10^6 cells/dish) and transfected

with 8 μg each of psiU6BX3.0-Mock (without insertion) and psiU6BX3.0-PRC1 (Si-#1, Si-#2, Si-#3 and two constructs [Si-m#1 and Si-m#2] including three-base or four-base substitutions in Si-#2) using the FuGENE6 transfection reagent (Roche) according to the supplier's recommendations. At 24 h after the transfection, cells are reseeded for RT-PCR (1×10^6 cells/10-cm dish), western blotting (1×10^6 cells/10-cm dish), colony formation assay (1×10^6 cells/10-cm dish) and MTT assay (2×10^5 cells/well). We selected the psiU6BX3.0-introduced T47D or HBC5 cells with medium containing 0.6 mg/mL or 0.4 mg/mL neomycin (Geneticin; Invitrogen, Carlsbad, CA, USA), respectively. We changed culture medium twice a week. To evaluate the knockdown effect of siRNA, we carried out semiquantitative RT-PCR using total RNAs extracted from the cells at 5-day incubation with neomycin, and western blot analysis with anti-PRC1 antibody using cell extracts harvested at 7-day incubation with neomycin. The specific primer sets for RT-PCR are as follows: 5′-GTGGTCCTAGGAGACTTGGTTTT-3′ and 5′-TACATGCATACCCCCAACAA-3′ for PRC1; and 5′-AACTTA-GAGGTGGGAGCAG-3′ and 5′-CACAACCATGCCTTACTT-TATC-3′ for *β2MG* as an internal control. Transfectants expressing siRNA were grown for 4 weeks in selective media containing neomycin, and then fixed with 4% paraformaldehyde for 15 min before staining with Giemsa solution (Merck, Whitehouse Station, NJ, USA) to assess colony number. To quantify cell viability, MTT assays were carried out using cell counting kit-8 according to the manufacturer's recommendation (Wako, Osaka, Japan). Absorbance at 570 nm was measured using a Microplate Reader 550 (Bio-Rad). These experiments were carried out in triplicate.

FACS analysis. The cell cycles of cultured T47D breast cancer cells were synchronized by treatment with 0.2 μg/mL aphidicolin (Sigma-Aldrich) for 16 h, followed by five PBS(–) washes and the addition of fresh culture media to release them from cell cycle arrest. After release from cell cycle arrest, the cells were collected and fixed with 70% ethanol, and then kept at 4°C before use. The cells were incubated with 10 mg/mL RNaseI in PBS(–) at 37°C for 30 min and stained with 50 μg propidium iodide at room temperature for 30 min. The cell suspensions at each time point were analyzed using FACscan (Becton Dickinson, Franklin Lakes, NJ, USA). Additionally, to detect the expression of endogenous PRC1 proteins in cells at various cell cycle points, we carried out western blot analysis using anti-PRC1 polyclonal antibody as described in the western blot analysis section.

Coimmunoprecipitation and immunoblotting analyses. COS-7 cells were transiently transfected with Flag-tagged PRC1V1 (pCAGGSn3FC-PRC1V1), HA-tagged KIF2C/MCAK (pCAGGS-nHA-KIF2C/MCAK) or both together using FuGENE6 transfection reagent. The cells were harvested 48 h after transfection, and were then lysed in lysis buffer. Equal amounts of total protein were incubated at 4°C for 1 h with either a rat anti-HA antibody (Roche) or a mouse anti-Flag antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunocomplexes were incubated with protein G-sepharose (Zymed Laboratories, South San Francisco, CA, USA) for 1 h at 4°C. After washing three times with lysis buffer, coprecipitated proteins were separated by SDS-PAGE and immunoblotted using either rat anti-HA or mouse anti-Flag antibodies.

Heterodimerization of three variants of the PRC1 protein. COS-7 cells transiently coexpressed combinations of the following constructs: Flag-tagged PRC1V1 (Flag-V1) and myc-tagged PRC1V2 (myc-V2) for the V1/V2 complex; myc-V2 and HA-tagged PRC1V3 (HA-V3) for the V2/V3 complex; and Flag-V1 and HA-V3 for the V1/V3 complex. Equal amounts of total protein were incubated at 4°C for 1 h with mouse anti-myc antibody 9E10 (Sigma-Aldrich) (V1/V2 and V2/V3), mouse anti-Flag antibody (Santa Cruz), or mouse anti-myc antibody 9E10 (Sigma-Aldrich) (V1/V3). Immunocomplexes were incubated with protein G-sepharose for 1 h at 4°C. After washing three times with lysis buffer, coprecipitated proteins were separated by SDS-PAGE and immunoblotted using a mouse anti-Flag antibody (V1/V2) or a rat anti-HA antibody (V2/V3 and V1/V3).

Statistical analysis. Statistical significance was determined by Student's *t*-test, using Statview 5.0 software (SAS Institute, Cary, NC, USA). A difference of $P < 0.05$ was considered to be statistically significant.

Results

Overexpression of PRC1 in breast cancers. We had previously carried out genome-wide gene expression profile analysis of 81 breast cancer cases using cDNA microarray representing 23 040 genes.[13] Among the genes upregulated in breast cancer cells, we focused on *PRC1* whose expression was significantly increased in the majority of breast cancer cases. We confirmed overexpression of PRC1 in 7 of 12 breast cancer cases by both cDNA microarray and semiquantitative RT-PCR analyses as shown in Fig. 1A. Upregulation of *PRC1* was observed in all 10 breast cancer cell lines examined (Fig. 1B), whereas the transcript was hardly detectable in normal human tissues except testis and thymus (Fig. 1C).

In the NCBI database, cDNA sequences corresponding to three transcriptional variants, denoted *PRC1* isoform 1 (*PRC1V1*) (GenBank accession no. NM_003981), *PRC1* isoform 2 (*PRC1V2*) (GenBank accession no. NM_199413) and *PRC1* isoform 3 (*PRC1V3*) (GenBank accession no. NM_199414), were deposited. The full-length cDNA sequences for the V1, V2 and V3 variants consist of 3128, 3091 and 3011 nucleotides that encode 620-, 606- and 566-amino acid peptides, respectively. These three transcriptional variants consist of 15, 14 and 14 exons, respectively; the V2 variant lacks exon 14 (42 nucleotides). The V3 variant that lacks 77 nucleotides corresponding to part of exon 13 and all of exon 14 has an early stop codon within the last exon (Fig. 1D). Subsequent semiquantitative RT-PCR experiments confirmed that all of the three variants were highly expressed in all 10 breast cancer cell lines examined in comparison with normal human mammary gland (Fig. 1E).

To investigate expression of endogenous PRC1 protein, we developed a polyclonal antibody to PRC1 that could recognize a region common to the three variants. Subsequent western blot analysis using cell lysates from 12 breast cancer cell lines (BT-20, HBC4, HBC5, HCC1937, MCF-7, MDA-MB-231, MDA-MB-435S, SKBR3, T47D, YMB-1, BT-474 and HBL100) as well as HMEC detected a high level of PRC1 expression in all of them, whereas the proteins were expressed at a low level in HMEC cells, and expression at the transcriptional level is undetectable in normal duct cells (Fig. 2A). Expectedly, we observed two bands, one corresponding to the predicted size of the V1 and V2 proteins (71.6 and 70.2 kDa in one band), and the other corresponding to the predicted size of the V3 protein (66.2 kDa), in all 12 of the breast cancer cell lines examined.

Furthermore, we examined the subcellular localization of endogenous PRC1 protein in breast cancer cell lines, HBC5, MCF-7 and T47D by immunocytochemical analysis using the anti-PRC1 polyclonal antibody. It was observed as a circular filament around interphase nuclei in these breast cancer cells (Fig. 2B, interphase). In prophase it was localized on the chromosomes, then with the entire mitotic spindle through metaphase into the early stage of anaphase. Finally, this protein was accumulated at the spindle midzone and midbody during anaphase to telophase in breast cancer cells (Fig. 2B, anaphase and telophase).

To further examine PRC1 expression in breast cancer and normal tissue sections, we carried out immunohistochemical staining with anti-PRC1 antibody and observed strong staining

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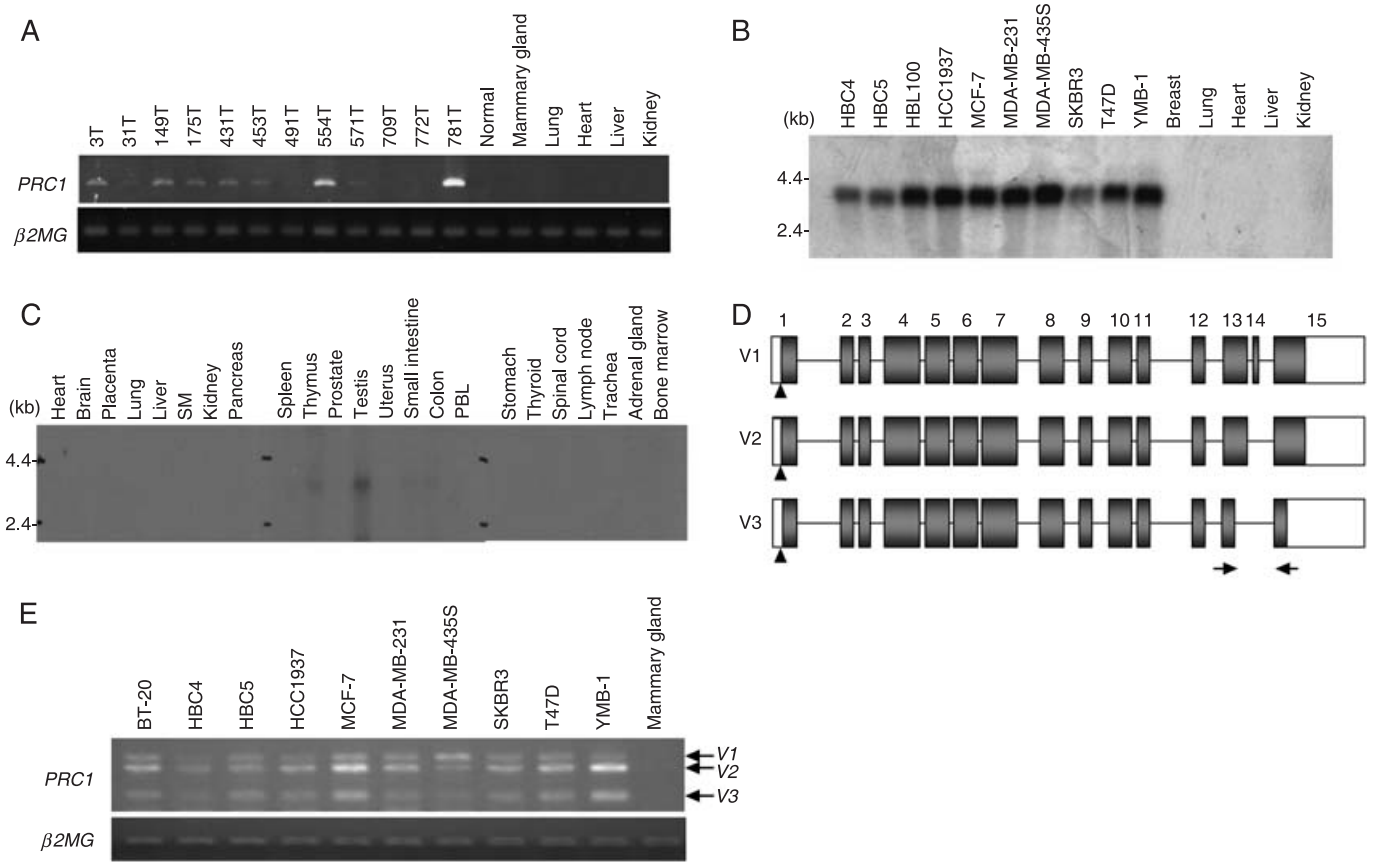

Fig. 1. Protein regulator of cytokinesis 1 (PRC1) overexpression in breast cancer cells. (A) Expression of PRC1 in microdissected breast cancer cases (3T, 31T, 149T, 175T, 431T, 453T, 491T, 554T, 571T, 709T, 772T and 781T) compared with normal human tissues (normal, microdissected normal breast ductal cells, mammary gland, lung, heart, liver and kidney) by semiquantitative reverse transcription–polymerase chain reaction. β2-Microglobulin (β2MG) served as a loading control. (B) Northern blot analysis of the protein regulator of cytokinesis 1 transcript in 10 breast cancer cell lines (HBC4, HBC5, HBL100, HCC1937, MCF-7, MDA-MB-231, MDA-MB-435S, SKBR3, T47D and YMB-1) and normal human tissues including breast, lung, heart, liver and kidney. A high-intensity band was observed in breast cancer cell lines, but no signal was detected in normal tissues. (C) Northern blot analysis of the PRC1 transcript in various human tissues. Bands are observed specifically in testis and thymus. (D) Genomic structure of three variants of PRC1 (V1, V2 and V3). Grey boxes indicate a coding region, and white boxes indicate non-coding regions. Black triangles indicate initiation codon. The arrows indicate a primer set to detect of each of the three variants. The number above each box indicates the exon number. (E) Expression pattern of each of the three PRC1 variants in 10 breast cancer cell lines and normal human mammary gland examined by semiquantitative RT-PCR. β2MΓ served as a loading control. PBL, peripheral blood leukocyte; SM, skeletal muscle.

in the cytoplasm and nuclei of three different histological subtypes of breast cancer: solid-tubular carcinoma (no. 234), papillotubular carcinoma (no. 240) and scirrhous carcinoma (no. 179) (Fig. 2C, first panels). Among the 53 breast cancer tissues examined, we observed strong staining in 38 cases (data not shown), confirming a high expression of PRC1 at the protein level. However, its expression was hardly detectable in normal mammary ductal cells (no. 10441). Furthermore, in concordance with the results of northern blot analysis, its expression was detected in the testis, whereas no expression was observed in heart, liver, kidney or lung (Fig. 2C, second and third panels).

Growth-inhibitory effects by siRNA to PRC1. To assess the growth-promoting role of PRC1, we knocked down the expression of endogenous PRC1 in the breast cancer cell lines T47D and HBC5, in which PRC1 was overexpressed, by means of the mammalian vector-based RNA interference technique. We designed three siRNA constructs targeted to the region common to the three variants. Semiquantitative RT-PCR analysis showed that PRC1-specific siRNAs (Si-#1 and Si-#2) significantly suppressed expression, compared with Mock or Si-#3 as controls (Fig. 3A). We then carried out MTT and colony-formation assays (Fig. 3B,C), and found that introduction of Si-#1 and Si-#2 constructs remarkably suppressed growth of both T47D and HBC5 cells (T47D: Si-#1, $P = 0.0008$ and Si-#2, $P = 0.0012$; HBC5: Si-#1, $P = 0.0095$ and Si-#2, $P = 0.007$; Student's *t*-test), in concordance with the results of knock-down effect. Furthermore, we used PRC1 siRNA (Si-#2) to confirm the reduction of PRC1 expression at the protein level by western blot analysis (Fig. 3D, third panel). To exclude the possibility of off-target effects by PRC1 siRNA (Si-#2), we generated two mismatched siRNAs, each of which contained a 3-bp or 4-bp replacement in Si-#2 (Si-m#1 and Si-m#2), and found that these mismatched constructs had no suppressive effect on PRC1 expression at the transcriptional or protein levels (Fig. 3D, first and third panels), or on the growth of HBC5 cells (Fig. 3E,F). These findings suggest that PRC1 is likely to play a critical role in the growth of these breast cancer cells.

Cell cycle-dependent expression of PRC1 variants in breast cancer cells. Because PRC1 is known to be a mitotic spindle-associated protein involved in cytokinesis,[17–19] we examined the amount of each of the three variant PRC1 proteins in various phases of the cell cycle by western blot analysis using breast cancer cell line T47D. FACS analysis showed that the proportion of cells at G_2/M phase was significantly increased (63.94%) at 9 h after release from cell cycle arrest (Fig. 4A). Interestingly, western blot analysis showed that the intensity of the two bands was

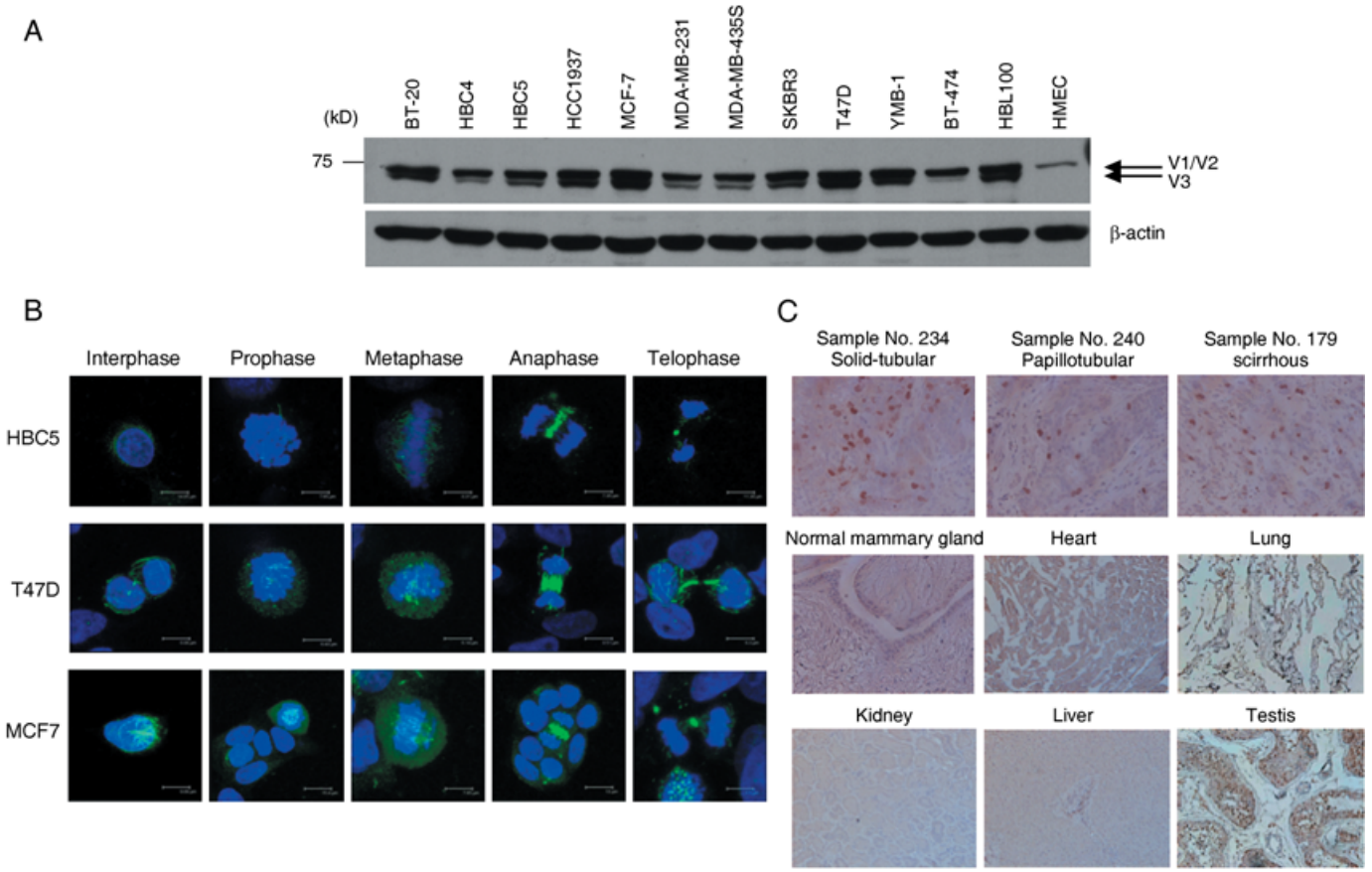

Fig. 2. Expression of protein regulator of cytokinesis 1 (PRC1) in breast cancer cell lines and tissue sections. (A) Expression of endogenous PRC1 protein in 12 breast cancer cell lines (BT-20, HBC4, HBC5, HCC1937, MCF-7, MDA-MB-231, MDA-MB-435S, SKBR3, T47D, YMB-1, BT-474 and HBL100) in comparison with the human mammalian epithelial cell (HMEC) cell line by western blot analysis using anti-PRC1 antibody. (B) Subcellular localization of endogenous PRC1 protein in breast cancer cells during the cell cycle. HBC5, T47D and MCF-7 cells were stained immunocytochemically using affinity-purified anti-PRC1 polyclonal antibody (red) and DAPI (blue) to discriminate the nucleus. (C) Representative images of immunohistochemical staining of PRC1 in breast cancer and normal tissue sections (normal mammary gland, heart, liver, kidney, lung and testis). Endogenous PRC1 protein was stained by anti-PRC1 antibody. Expression was hardly detectable in a normal breast tissue (sample no. 10441), but cancer cells were stained strongly in all cancer tissues investigated: solid-tubular (sample no. 234), papillotubular (sample no. 240) and scirrhous (sample no. 179) carcinomas. Original magnification × 100. V1, PRC1 variant 1; V2, PRC1 variant 2; V3, PRC1 variant 3.

increased at 3, 6 and 9 h after release from cell cycle arrest, but decreased at the 12-h and 15-h time points (Fig. 4B). In particular, V3 protein expression was significantly elevated during the 3–9-h time points. Taken together, the amounts of V1 and V2, and V3 protein were remarkably increased in G_2/M phase breast cancer cells, suggesting an important role for each PRC1 variant in cell division.

Interaction of PRC1 with KIF2C/MCAK. PRC1 was reported to interact with KIF4, KIF14 and MgcRacGAP.[20–23] However, because these three molecules were not expressed in breast cancer cells in our microarray data (data not shown), we attempted to identify a molecule that interacts with PRC1 in breast cancer cells. We focused on KIF2C/MCAK protein because this protein is known to be involved in mitosis and its expression pattern was very similar to *PRC1* in our expression profile analysis of breast cancer.[13] We first compared the expression patterns of PRC1 and KIF2C/MCAK by semiquantitative RT-PCR analysis and confirmed co-overexpression of PRC1 and KIF2C/MCAK in breast cancer cell lines (Fig. 5A). To investigate an interaction between PRC1 and KIF2C/MCAK proteins, we constructed plasmids designed to express Flag-tagged PRC1V1 (PRC1-Flag) and HA-tagged KIF2C/MCAK (KIF2C-HA). These plasmids were cotransfected into COS-7 cells, and then the proteins were immunoprecipitated with anti-HA antibody. Immunoblotting of the precipitates using anti-Flag antibody indicated that KIF2C-HA was coprecipitated with PRC1-Flag (Fig. 5B, left panel). Conversely, we carried out immunoprecipitation using anti-Flag antibody and then immunoblotting of the precipitates using anti-HA antibody. The results showed that PRC1-Flag was also coprecipitated with KIF2C-HA (Fig. 5B, right panel). We also observed an interaction between exogenously expressed PRC1-V2 and -V3 proteins and KIF2C/MCAK, as well as the PRC1V1 protein (data not shown).

Discussion

Through identification and characterization of molecules specifically expressed in cancer cells, molecular-targeting drugs for cancer therapy have been developed in the last decade. However, the proportion of patients showing good response to presently available treatments is still very limited.[3] Hence, there is an urgent need to discover good molecular targets that may be used in the development of new anticancer agents. In the present report, through the comprehensive expression profile analysis of clinical breast cancer tissues,[13] we identified *PRC1* as being significantly overexpressed in both clinical breast cancer samples and breast cancer cell lines. Furthermore, northern blot analysis revealed that *PRC1* expression was barely detectable in any normal human tissues except the testis and thymus. Immunohistochemical staining experiments using

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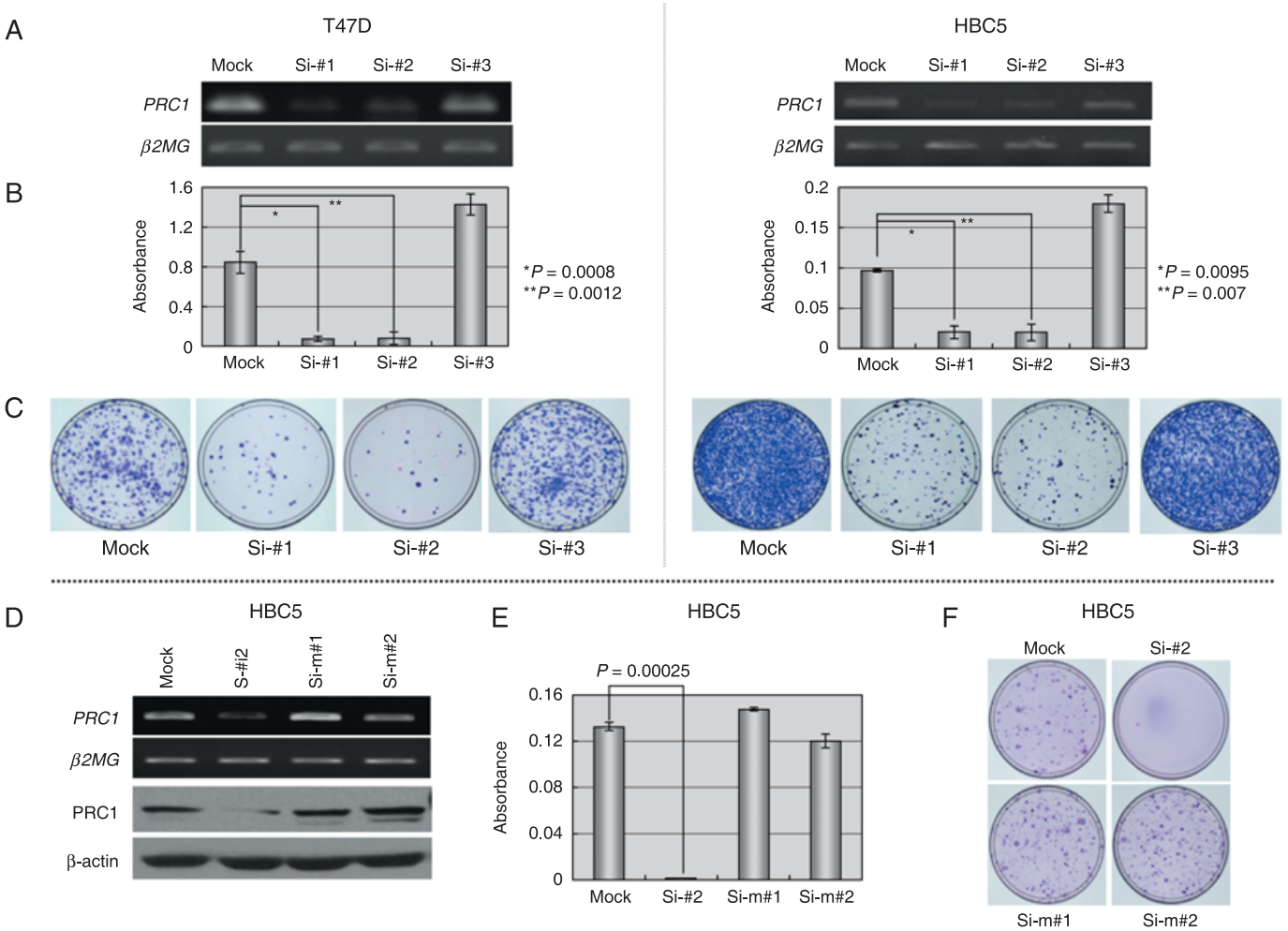

Fig. 3. Growth-inhibitory effects of protein regulator of cytokinesis 1 (PRC1) small interfering RNA (siRNA) to breast cancer cells. (A) Semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) showing suppression of endogenous expression of PRC1 by PRC1-specific siRNA (Si-#1, Si-#2 and Si-#3) in breast cancer cell lines T47D (left panel) and HBC5 (right panel) cells. Mock-siRNA (Mock) was used as a control. β2-Microgloblin (β2MG) was used as a quantitative control for RT-PCR. (B) MTT assay demonstrating a decrease in the numbers of colonies by knockdown of PRC1 in T47D (left panel: Si-#1 $P = 0.0008$ and Si-#2 $P = 0.0012$, respectively; unpaired *t*-test) and HBC5 (right panel: Si-#1 $P = 0.0095$ and Si-#2 $P = 0.007$, respectively; unpaired *t*-test) cells. (C) Colony-formation assay demonstrating a decrease in the number of colonies by knockdown of PRC1 in T47D (left panels) and HBC5 (right panels) cells. (D) PRC1 siRNA (Si-#2) designed to knock down expression of PRC1 at the transcriptional and protein levels in HBC5 cells, whereas mismatched PRC1 siRNA (Si-m#1 and Si-m#2) did not reduce PRC1 expression by semiquantitative RT-PCR (first and second panels) or western blot (third and forth panels) analyses (E) by MTT assay (Si-#2, $P = 0.00025$; unpaired *t*-test) and (F) by colony-formation assay.

anti-PRC1 polyclonal antibody clearly indicated upregulation of PRC1 expression in breast cancer cells, but no expression in surrounding normal cells or in vital organs (Fig. 2C).

We also demonstrated that knockdown of endogenous PRC1 by siRNAs caused dysfunction in the cytokinesis process in breast cancer cells and resulted in cell death (Fig. 3). These findings suggest that *PRC1* might play a critical role in cytokinesis of breast cancer cells. However, the NIH3T3-derivative cells in which stable expression of *PRC1* was introduced did not show any growth enhancement (data not shown), suggesting that *PRC1* is essential for the survival of breast cancer cells, but not sufficient for enhancing cell growth. Furthermore, our cDNA microarray data indicated upregulation of *PRC1* in almost all clinical cancers we examined; these included cholangiocarcinomas, colon cancers, non-small cell lung cancers and pancreatic cancers as well as breast cancers.[24–27] These results showed that this gene should serve as a valuable target for the development of anticancer agents for many types of human cancer.

Due to its functional similarity and coexpression in breast cancer cells, we focused on KIF2C/MCAK as a candidate PRC1-interacting protein. We confirmed the *in vivo* interaction of PRC1 and KIF2C/MCAK, and that knockdown of KIF2C/MCAK expression with KIF2C/MCAK-specific siRNAs also suppresses growth of breast cancer cells because of the failure of cytokinesis, similar to PRC1 (data not shown). PRC1, a mitotic spindle-associated Cdk substrate, is a microtubule-binding and -bundling protein, and organizes midzone formation that is necessary for cytokinesis.[18,21] KIF2C/MCAK, a member of the KinI subfamily of kinesin-related proteins, contains the motor domain in its middle region[28] and regulates microtubule length in cell division[29] and interphase microtubule dynamics.[30] Because PRC1 is not a microtubule-based motor protein, movement of PRC1 along the spindle during mitosis and cytokinesis in breast cancer cells might be regulated by the KIF2C/MCAK protein. This evidence suggests a crucial role for the PRC1–KIF2C/MCAK complex in mammary carcinogenesis, particularly in the cell division process of cancer cells.

Furthermore, we demonstrated that all PRC1 variants were overexpressed in breast cancer cells (Fig. 1E), and that there was a significant increase in endogenous PRC1 protein level

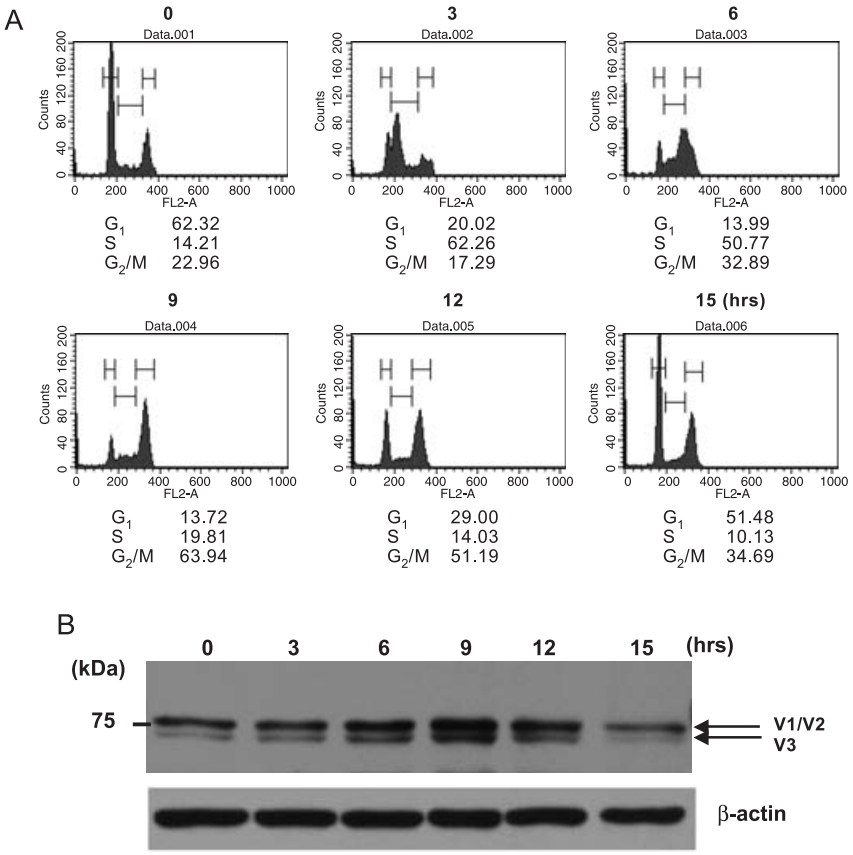

Fig. 4. Expression of protein regulator of cytokinesis 1 (PRC1) variant proteins during mitosis (V1, V2, V3). (A) Fluorescence-activated cell sorting (FACS) analysis showing a population of T47D cells collected every 3 h from 0 to 15 h after synchronization. (b) Western blot analysis of PRC1 during mitosis in T47D cells. It is notable that expression of PRC1 was upregulated at 3–9 h after release from cell cycle arrest when the majority of cells were at G_2/M phase.

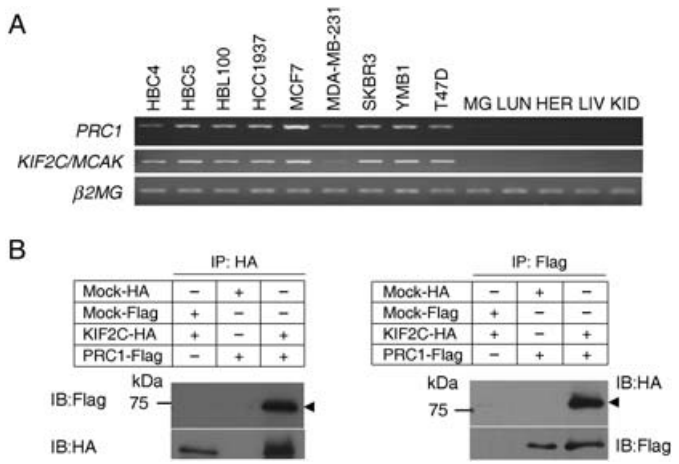

Fig. 5. Interaction between protein regulator of cytokinesis 1 (PCR1) and kinesin family member 2C/mitotic centromere-associated kinesin (KIF2C/MCAK). (A) Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) experiments for PRC1 and KIF2C/MCAK transcripts in nine breast cancer cell lines (HBC4, HBC5, HBL100, HCC1937, MCF-7, MDA-MB-231, SKBR3, YMB1 and T47D) and normal human tissues (mammary gland, lung, heart, liver and kidney). β2-Microglobulin (β2MG) was used as a quantitative control. (B) Coimmunoprecipitation of PRC1 and KIF2C/MCAK. Cell lysates from cos-7 cells transfected with flag-tagged PRC1 and hemagglutinin (HA)-tagged KIF2C/MCAK proteins were immunoprecipitated with either anti-Flag monoclonal or anti-HA antibodies. Immunoprecipitates were immunoblotted using monoclonal anti-HA or anti-Flag antibodies. IP, immunoprecipitation.

in G_2/M phase by western blot analysis (Fig. 4). PRC1 was reported to form homo-oligomers through its N-terminal region (codons 1–184 of the V1 protein) and its oligomerization was shown to be essential for midzone formation in late mitosis and cytokinesis.[31] Because these three PRC1 variants commonly contain the N-terminal oligomerization domain, we examined the possibility of heterodimerization of these variants in breast cancer cells by coimmunoprecipitation analysis and found the formation of heterodimers of all three combinations: V1/V2, V2/V3 and V1/V3 (Supplementary Fig. 1). Hence, the heterocomplex formation of these PRC1 variants might play an important role in cell division in breast cancer cells, although the physiological role of each of these complexes is still unclear.

In conclusion, our findings clearly suggest that PRC1 is overexpressed in breast cancer cells, and the formation of a complex between PRC1 and KIF2C/MCAK is likely to play a significant role in cytokinesis of these cells. Recent anticancer drug development is focused on targeting important molecules involved in oncogenic pathways. We found that downregulation of *PRC1* by treatment with siRNA significantly suppresses the growth of breast cancer cells, indicating its crucial role in proliferation of breast cancer cells. Our data should contribute to a better understanding of mammary carcinogenesis, and indicates that PRC1 is a promising molecular target for breast cancer treatment.

Acknowledgments

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Supplementary Material

The following supplementary material is available for this article:

Supplementary Fig. 1. Heterodimerization of three variants of protein regulator of cytokinesis 1 (PRC1) protein. COS-7 cells were transiently coexpressed with Flag-V1 and myc-V2 (V1/V2), myc-V2 and HA-V3 (V2/V3), or Flag-V1 and HA-V3 (V1/V3). Coimmunoprecipitation was carried out using anti-myc antibody (V1/V2 and V2/V3) and anti-Flag-antibody (V1/V3). Immunoblotting analysis was performed using anti-Flag antibody (V1/V2) or anti-HA antibody (V2/V3 and V1/V3). HA, hemagglutinin; IB, immunoblot; V1, PRC1 variant 1; V2, PRC1 variant 2; V3, PRC1 variant 3.

Fig. S1. Hetero-dimerization of three variants of PRC1 protein. COS-7 cells were transiently coexpressed with Flag-V1 and myc-V2 (V1/V2), myc-V2 and HA-V3 (V2/V3), or Flag-V1 and HA-V3 (V1/V3). Co-immunoprecipitation was performed using anti-myc antibody (V1/V2 and V2/V3) and anti-Flag-antibody (V1/V3), respectively. Immunoblot analysis was performed using anti-Flag antibody (V1/V2) or anti-HA-antibody (V2/V3 and V1/V3).

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