Human Cancer Biology

Decreased Poly(ADP-Ribosyl)ation of CTCF, a Transcription Factor, Is Associated with Breast Cancer Phenotype and Cell Proliferation

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Abstract

Purpose: There is compelling evidence of a relationship between poly(ADP-ribosylation) and tumorigenesis; however, much less is known about the role of specific targets of poly(ADP-ribosylation) in tumor development. Two forms of the multifunctional transcription factor, CTCF, were previously identified: a 130-kDa protein (CTCF-130), characteristic for cell lines, and a 180-kDa protein (CTCF-180), modified by poly(ADP-ribosylation). This study was aimed to investigate differential poly(ADP-ribosylation) of CTCF in normal and tumor breast tissues.

Experimental Design: Western blot analysis, mass spectrometry, and immunohistochemical and immunofluorescent stainings were used to characterize CTCF-130 and CTCF-180 in breast cell lines, primary cultures, and normal and tumor breast tissues. The immunoreactivity score was used for CTCF-130 quantification in tissues.

Results: We discovered that only CTCF-180 is detected in the normal breast tissues, whereas both CTCF-130 and CTCF-180 are present in breast tumors. Using an antibody specific for CTCF-130, we observed that 87.7% of breast tumors were positive for CTCF-130. A negative correlation existed between the levels of CTCF-130, tumor stage, and tumor size. Significantly, a transition from CTCF-180 to CTCF-130 was discovered in primary cultures generated from normal breast tissues, indicating a link between CTCF-130 and proliferation. Conversely, the appearance of CTCF-180 was observed following growth arrest in breast cell lines.

Conclusions: Collectively, our data suggest that the loss of CTCF poly(ADP-ribosylation) is associated with cell proliferation and breast tumor development. We propose the use of CTCF-130 as a marker for tumor breast cells and lower levels of CTCF-130 as an indicator of unfavorable prognosis. (Clin Cancer Res 2009;15(18):5762–71)

CTCF is a conserved and ubiquitous 11–zinc finger transcription factor with features of a tumor suppressor protein (1, 2). The important role of CTCF in breast tumorigenesis, which includes both genetic and epigenetic components, has been documented (3–6). CTCF regulates various cellular processes such as growth, differentiation, apoptosis, imprinting, and X chromosome inactivation, exerting its functions through various mechanisms including gene activation, repression, silencing, and control of insulator elements (refs. 1, 2, 5, 6, and references therein). Paradoxically, and perhaps controversially, high CTCF levels in breast cancer cells are linked to an antiapoptotic function of CTCF (7).

CTCF functions are modulated by post-translational modifications, one of which is poly(ADP-ribosylation) (8, 9). The general ADP-ribosylation reaction is catalyzed by the enzymes poly(ADP-ribose) polymerases (PARP); the reaction involves transfer of ADP-ribose residue of NAD+ to acceptor protein (10). The PARP family includes several members, but the best studied form, PARP-1, is shown to play a role in DNA repair, recombination, cell proliferation, cell death, genomic stability, and...
cancer (11, 12). The dynamic metabolism of the poly(ADP-ribose) polymer (PAR) has been well established: PAR can be rapidly hydrolyzed by poly(ADP-ribose) glycohydrolase (13) and ADP-ribosyl protein lyase (14).

Previous studies revealed that loss of poly(ADP-ribosyl)ation is linked to tumor development; in this context, PARP-1 is regarded as a tumor suppressor protein (ref. 12, and references therein). However, the exact molecular mechanisms, particularly the role(s) of specific targets of poly(ADP-ribosyl)ation in tumor development, are not well understood. A growing body of evidence suggests a connection between poly(ADP-ribosyl)ation of nuclear proteins and regulation of transcription of various genes linked to tumorigenesis (ref. 10, and references therein).

Given the importance of both CTCF and PARPs, the investigation of the relationship between these proteins in normal development and disease, particularly tumorigenesis, is of great interest. We reported previously that poly(ADP-ribosyl)ation of CTCF plays a key role in the regulation of the insulator function of CTCF (8, 9). Conversely, activation of PARP-1 by CTCF has been shown to lead to inhibition of DNA methyltransferase and DNA hypomethylation (15). Notably, CTCF and PARP-1 can interact in vivo and in vitro (15, 16), which may be an important consideration in explaining the CTCF-PARP-1 regulatory circuit.

Using biochemical assays, such as Western blot analysis, it is possible to distinguish the non-poly(ADP-ribosyl)ated and poly(ADP-ribosyl)ated forms of CTCF. In cultured cells, the majority of the CTCF pool is represented by a 130-kDa protein, the non–poly(ADP-ribosyl)ated form of CTCF (CTCF-130; refs. 7, 8, 17). A minor band of the highly poly(ADP-ribosyl)ated form of CTCF, migrating as a 180-kDa protein (CTCF-180), can also be detected in some cases (8).

In this study, we investigated the possible link between CTCF poly(ADP-ribosyl)ation and breast tumorigenesis. We discovered that the sole presence of CTCF-180 is characteristic for normal breast tissue samples, whereas CTCF-130 can be only detected in breast tumor tissues. Our data also provide evidence that the two forms of CTCF may be linked to different functions of CTCF in nondividing and proliferating breast cells.

### Materials and Methods

#### Human tissues.
Primary human tumor tissues together with paired peripheral tissues (also called “normal”) were collected during surgery from breast cancer patients treated at Colchester General Hospital, with written consent taken before surgery. The study was approved by the local ethics committee (reference number MH363). Tissue samples were also collected after breast reduction surgery. Tissue specimens were visually examined by an experienced pathologist as fresh material, tumor tissues were selected by conventional pathologic criteria, and the histopathology was further confirmed by microscopic examination. To obtain primary cultures, fresh tissues were immediately processed (see below); portions of the specimens were frozen and stored at -80°C. The normal tissues from other organs were obtained from IAP Tissue Bank in Lausanne, Switzerland, with the approval from the Swiss Ethics Commission.

#### Cells.
Breast cancer cell line MCF7 was maintained in RPMI 1640 with Ultraplumatinine 1 (Lonza) supplemented with 10% fetal bovine serum (Biosera) and 50 μg/mL gentamicin (Life Technologies/Invitrogen). The immortalized normal luminal cell line 226LDM was generated using the viral constructs carrying the modified T antigen, TAg(U19dl97-98) (18), and hTERT and will be described elsewhere. This cell line was grown in DMEM/F-12 (Lonza) supplemented with 5 μg/mL insulin, 1 μg/mL hydrocortisone, 20 μg/mL epidermal growth factor, 20 μg/mL cholera toxin (all from Sigma), 10% fetal bovine serum, and 50 μg/mL gentamicin. For induction of growth arrest, MCF7 cells were grown in RPMI 1640 without t-glutamine and phenol red (Lonza) supplemented with 5% charcoal-stripped fetal bovine serum (PAA); 226LDM cells were cultured in complete growth medium. Standard methods were used to induce growth arrest in S and G2-M phases (19–21). Cells were blocked in S phase by treatment with either hydroxyurea alone or hydroxyurea combined with thymidine for 48 h at the specified concentrations. To induce growth arrest in G2-M phase, cells were first cultured for 48 h with either hydroxyurea alone or hydroxyurea combined with thymidine released from the drugs for 1 h, and then treated with 500 ng/mL nocodazole for 24 h. All chemicals were from Sigma. To achieve growth arrest in MCF7 cells, the cells were treated with 5 mmol/L sodium butyrate for varied times, ultimately to induce apoptosis.

#### Primary breast cell cultures.
To obtain primary cells from fresh paired peripheral and tumor tissues, we essentially used a method described earlier (22) with modifications (23). Immunochemistry, immunofluorescence, and proliferation assay.
Immunohistochemical analysis of frozen breast tissue sections was done by staining with the Vectastain Elite ABC standard kit (Vector Laboratories) as suggested by the manufacturer. CTCF expression was analyzed using the primary mouse monoclonal anti-CTCF isotype IgG1 (BD Biosciences) and rabbit polyclonal anti-CTCF (Abcam) antibodies at 1:50 and 1:100 dilution, respectively. The secondary goat biotinylated anti-mouse and anti-rabbit antibodies were from Vector Laboratories; both were used at 1:200 dilution. The sections were then counterstained with H&E.

The immunologic CTCF staining was evaluated using the immunoreactivity score (IRS) as described previously (24). In brief, the percent- age of CTCF-positive cells was divided into four categories: (a) <10%, (b) 11% to 50%, (c) 51% to 80%, and (d) >80%, whereas the staining intensity was given a scale from 0 (no detectable immunostaining) to 3 (strong immunostaining). The IRS (0-12) was then calculated by multiplying the score values. Several fields of view and a minimum of 400 cells were counted. Scoring was done in a blinded fashion by

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two independent scorers, with each slide read twice; the IRS results represent the average score for each sample.

For double-immunofluorescent staining of frozen breast tissue sections and primary breast cells, standard protocols were used (7). CTCF was analyzed with the mouse monoclonal anti-CTCF isotype IgG1 (BD Biosciences) and rabbit polyclonal anti-CTCF (Abcam) antibodies at 1:50 and 1:100 dilution, respectively. The histologic type of cells was determined using the following primary antibodies: mouse anti-CK14 antibody, isotype IgG3, specific for myoepithelial cells (clone LL002;Cloneix International) at 1:10 dilution, and mouse anti-CK19, isotype IgG2a, specific for luminal cells (clone LP2K; personal gift from Prof. Lane, University of Dundee; refs. 25, 26) at 1:2 dilution. The fluorescent-conjugated secondary antibodies used in the study were as follows: goat anti-rabbit IgG FITC (Abcam) at 1:250 dilution and goat anti-mouse IgG-specific FITC, goat anti-mouse IgG-specific TRITC, and goat anti-rabbit IgG-specific TRITC (all anti-IgG specific antibodies were from Southern Biotech); all were diluted at 1:200 dilution.

Proliferation assay was done using the 5-bromo-2’-deoxyuridine labeling and detection kit I (Roche) according to the manufacturer’s instructions. Nuclei were stained with 4′,6-diamidino-2-phenylindole. The prepared samples were mounted in anti-fading medium containing MOWIOL (Mowiol 4-88; Calbiochem) and DABCO (Sigma).

Confocal images were obtained using a Bio-Rad Radiance 2000 confocal unit on an Olympus IX70 microscope. The Olympus BX41 microscope was employed for bright-field and fluorescent imaging, for the latter with fluorescence filter sets XF06 (4′,6-diamidino-2-phenylindole), XF100-2 (FITC), and XF33 (TRITC; Omega Optical). Images were acquired by a Colorview II camera (Soft Imaging Systems).

**Western blot analysis.** The lysates for Western blot analysis were prepared from cells as described in Klcnova et al. (17) and from tissues as described in D’Arcy et al. (23). Western blot assays were conducted as described previously (8) using the following primary antibodies: polyclonal anti-CTCF (Abcam), monoclonal anti-CTCF (BD Biosciences), anti-α-tubulin (Sigma), anti-PARP (Calbiochem), and anti-PAR 10H (Alexis Biochemicals). The conjugated secondary antibodies, anti-rabbit and anti-mouse horseradish peroxidase, were purchased from Abcam. Detection was done with enhanced chemiluminescence reagent (GE Healthcare) according to the manufacturer’s instructions.

**Immunoprecipitation experiments and mass spectrometry.** Immunoprecipitation experiments were done as described previously (23) using the polyclonal rabbit anti-CTCF antibody (Abcam). Mass spectrometry analysis was done as described earlier (23). In brief, the band of interest was excised and subjected to the in-gel tryptic digestion, and the tryptic peptides were subsequently extracted and analyzed using liquid chromatography-tandem mass spectrometry (Bruker Esquire). Results were analyzed using the in-house built Mascot Server.

**Flow cytometry analysis.** The distribution of cells at specific cell cycle stages was evaluated by flow cytometry. The protocol was carried out according to a previously described method (27). A proportion of cells were used to prepare lysates for Western blot analysis, whereas the remaining fraction was analyzed by fluorescence-activated cell sorting using Becton Dickinson FACSCalibur cell sorter and the CellQuest software. Each fluorescence-activated cell sorting analysis sample contained 3 x 10^6 cells.

**Statistical analysis.** Statistical analysis was carried out using unpaired Student’s t test. A significant value was detected when the probability was below the 5% confidence level (P ≤ 0.05).

**Results**

**Different forms of CTCF are present in normal and tumor breast tissues.** In our initial experiments, we assessed the CTCF protein levels in 64 normal and tumor paired breast tissues by Western blot analysis using the rabbit polyclonal anti-CTCF antibody (“anti-CTCF poly”; Abcam). Surprisingly, the results of this analysis revealed that a slow migrating form of CTCF was present as the sole band in all 64 paired peripheral (“normal”) breast tissue samples tested. Representative Western blots of four paired tissues and eight additional paired tissues are shown in Fig. 1A (top) and Supplementary Fig. S1, respectively. A form of CTCF with similar characteristics was observed in our previous studies and described as a highly poly(ADP-ribosyl)ated form of CTCF (CTCF-180; ref. 8).

In contrast, in breast tumor tissue samples, in addition to the slower migrating form, the faster migrating form of CTCF (CTCF-130), observed previously in all breast cell lines (7), was detected (Fig. 1A). Of note, the total level of CTCF in breast tumors was elevated in comparison with the normal tissues, thus confirming our previous observations (7). To investigate whether the slow migrating form of CTCF detected in breast tissues exhibits the same biochemical features as the previously described CTCF-180 (8), we used lysates from normal breast tissues for immunoprecipitation with the anti-CTCF rabbit polyclonal antibody (“CTCF poly”) followed by Western blot analysis. Normal breast tissues were chosen for these experiments, as they only contain the slow migrating form of CTCF. Western blot analysis of the immunoprecipitated proteins with the anti-CTCF and the anti-PAR antibodies revealed that the band of 180 kDa was recognized by both antibodies (Fig. 1B, top), indicating that this is a poly(ADP-ribosyl)ated form of CTCF. To confirm the identity of the 180-kDa protein, the 180-kDa band resolved after immunoprecipitation with the anti-CTCF polyclonal antibody was excised from the Coomassie stained SDS-PAGE gel (Fig. 1B, bottom). The gel slice was subjected to the “in-gel” digestion, and peptides were subsequently extracted and analyzed by mass spectrometry. The database interrogation of the recovered peptide sequences matched human CTCF (Swiss-Prot protein database accession number P49711); these peptide sequences are presented in Fig. 1B (bottom, box on the right). From these experiments, we concluded that the slower migrating form of CTCF detected in breast tissues is the poly(ADP-ribosyl)ated form of CTCF (CTCF-180).

We then investigated if the presence of only CTCF-180 was specific for normal breast tissues or it was also a feature of all normal tissues from other organs. For this purpose, lysates of normal tissues from different organs (spleen, rectum, colon, liver, stomach, uterus, and pancreas) were prepared and analyzed by Western assay. As shown in Fig. 1C (top), CTCF-180 was detected in all specimens. In contrast with normal breast tissues, the CTCF-130 form was revealed in these normal tissue specimens when using the monoclonal antibody, specifically recognizing CTCF-130 (see next section; Fig. 1C, middle). However, CTCF-130 was not detected in some of the tissues with the anti-CTCF “poly” antibody due to a smaller amount of protein, as evident from the tubulin signal (Fig. 1C, bottom).

**Anti-CTCF monoclonal antibody (BD Biosciences) specifically recognizes only CTCF-130.** To identify an antibody that could specifically recognize only one of the forms of CTCF, we used Western blot analysis to screen a panel of anti-CTCF antibodies available commercially. In this analysis, the anti-CTCF monoclonal antibody (or anti-CTCF “mono”) from BD Biosciences was found to specifically recognize only CTCF-130 (Fig. 1A and C). Interestingly, this antibody showed a much higher affinity to CTCF-130 than the polyclonal antibody, as it detects lower amounts of CTCF-130 (Fig. 1C).
The specificity of the anti-CTCF monoclonal antibody to CTCF-130 was also tested by immunohistochemical staining of sections from normal and tumor breast tissues (Fig. 2A). In agreement with the previously published data (7), in normal breast tissues, a weak positive staining, both cytoplasmic and nuclear, could be detected with the anti-CTCF "poly" antibody. The specificity of this antibody was confirmed using the pre-blocked antibody, which resulted in abrogation of the staining (Supplementary Fig. S2).

In contrast, normal breast tissues probed with the anti-CTCF "mono" antibody did not reveal any positive staining. In the paired tumor tissues, strong signals, predominantly nuclear,
were observed with both anti-CTCF “mono” and anti-CTCF “poly” antibodies (Fig. 2A). The results of immunohistochemical staining with these antibodies were further confirmed by immunofluorescent staining (see below).

Collectively, the data described in this section show that the anti-CTCF “mono” antibody can specifically recognize CTCF-130.

CTCF-130 is detected in 87.7% of breast tumors and levels of CTCF-130 negatively correlate with tumor size, stage, and lymph node status. We next used a panel of breast reduction, paired peripheral and tumor tissues to quantify the levels of CTCF-130 by calculating the IRS values following immunohistochemical staining with the anti-CTCF “mono” antibody. These results are presented in Supplementary Table S1 and summarized in Fig. 3A. The IRS for CTCF-130 was zero in the breast reduction tissue, whereas the average IRS for the eight paired peripheral-to-tumor tissues tested was 0.25 ± 0.088 due to one weakly positive specimen. In breast tumors, CTCF-130 was detected in 43 of 49 (87.7%) samples, with an average IRS of 3.35 ± 0.049 (“All Phenotype group”). Examples of tumors with different IRS immunostained with the anti-CTCF “mono” antibody are given in Fig. 2B. Notably, the staining for CTCF-130 is heterogeneous in different tissue specimens, with different proportions of CTCF-130-positive tumor cells and a different degree of CTCF-130 levels in the specimens (Fig. 2B; Supplementary Table S1).

![Fig. 3. Correlation between levels of CTCF-130 and clinicopathologic characteristics of breast cancer patients. A, CTCF-130 levels were assessed using immunohistochemical staining for different breast tumor phenotypes. B, CTCF-130 levels were assessed using immunohistochemical staining in the entire tumor group; all phenotypes were included in the analysis. The immunohistochemical staining was quantified using the IRS and calculated by multiplying the percentage of stained cells and staining intensity as described previously (ref. 25; see Materials and Methods for details). The numbers of samples are shown above the bars. A, analysis of CTCF-130 levels in breast reduction tissues, paired peripheral tissues, and tumors with different phenotypes. Immunoreexpression of CTCF-130 is presented as mean IRS ± SE. The IRS value for CTCF-130 was 3.35 ± 0.049 in “All Phenotype group (ALL).” The IRS values for CTCF-130 in different groups were 4.75 ± 0.75 in ductal carcinomas in situ (DCIS), 2.96 ± 0.098 in invasive ductal carcinomas (IDC), 3.71 ± 0.198 in invasive lobular carcinomas (ILC), 4 ± 0 in mixed tumors (mixed), and 6 ± 2.12 in mucinous carcinomas (Muc Ca). The difference in IRS between the tumors and the combined group of paired peripheral (PP) and breast reduction (BR) tissues was significant for all groups: ductal carcinomas in situ, P ≤ 0.0004; invasive adenocarcinomas, P ≤ 0.0002; invasive ductal carcinomas, P ≤ 0.002; invasive lobular carcinomas, P ≤ 0.0005; mixed tumors, P ≤ 0.0002; mucinous carcinomas, P ≤ 0.0005; and “All tumors,” P ≤ 0.0001, unpaired Student’s t test. B, correlation between CTCF-130 levels in breast tumors and tumor size. Breast tumors were grouped according to their size; immunoreexpression of CTCF-130 in these groups is presented as mean IRS ± SE. Three groups of tumor sizes were analyzed: 10 to 19, 20 to 29, and >30 mm in diameter. The mean IRS for CTCF-130 in the first group of tumors (10-19 mm in size) was 4.44 ± 0.24. The mean IRS values decreased progressively with increasing tumor size, achieving 3.12 ± 0.16 in patients with tumor size 20 to 29 mm and 2.9 ± 0.1 in patients with tumor size >30 mm. Statistical evaluation of this data showed significant differences between CTCF-130 levels between the first and the second groups (P ≤ 0.0001) and between the first and the third groups (P ≤ 0.004). No significant difference was observed between the second and the third groups. C, correlation between CTCF-130 levels in breast tumors and tumor stage. Breast tumors were grouped according to their stage (I, II, and III); immunoreexpression of CTCF-130 in these groups is presented as mean IRS ± SE. The mean IRS values were 5.14 ± 0.39 for the group with stage I, 2.97 ± 0.07 for the group with stage II, and 2.43 ± 0.38 for the group with stage III. Statistical evaluation of the data showed significant differences between CTCF-130 levels between groups with stage I and II (P ≤ 0.01) and between groups with stage I and III (P ≤ 0.03). No significant difference was observed between the groups with stage II and stage III. D, correlation between CTCF-130 levels in breast tumors and lymph node metastasis status. Breast tumors were grouped according to the lymph node metastasis status: negative (neg) or positive (pos); immunoreexpression of CTCF-130 in these groups is presented as mean IRS ± SE. The mean IRS value was 3.04 ± 0.1 for the group of patients with positive lymph node metastasis and 3.9 ± 0.12 for the group of patients with negative lymph node metastasis. This difference was not statistically significant (P ≤ 0.17).
We also investigated a possible correlation between CTCF-130 levels in breast tumor tissues and clinicopathologic parameters: tumor stage, tumor grade, tumor size, estrogen receptor status, progesterone receptor status, HER-2 receptor status, status of auxiliary lymph nodes, preoperative and postoperative chemotherapy, menopausal state, and patient's age. A negative correlation was identified between the levels of CTCF-130 and tumor size (Fig. 3B) and tumor stage (this parameter reflects the extent of cancer growth; Fig. 3C). Lower mean IRS values were observed in patients positive for lymph node metastasis (IRS = 3.04 ± 0.1) compared with the group negative for lymph node metastasis (IRS = 3.9 ± 0.12; Fig. 3D); however, this difference was not statistically significant (P ≤ 0.17). No correlations with other parameters were found in these studies.

From these experiments, we concluded that CTCF-130, the "tumor-specific" form of CTCF, has characteristic features and potential as a breast cancer biomarker; this aspect is further explored in Discussion.

**Distribution of CTCF-130 and CTCF-180 in normal and tumor breast tissues.** We further investigated the patterns of distribution of CTCF-130 and CTCF-180 in normal and tumor breast tissues using immunofluorescent staining with markers specific for different cell types: CK14 for myoepithelial cells (28) and CK19 for luminal cells (29). Staining of normal breast tissues with the anti-CTCF "poly" antibody revealed that both myoepithelial cells and luminal cells contained CTCF-180 (Fig. 4A). On the other hand, no signal was detected for CTCF-130 when the anti-CTCF polyclonal antibody was used (Fig. 4A), confirming the results obtained by immunohistochemical staining (Fig. 2A; Table 1).

The same combination of the antibodies was used for immunofluorescent staining of breast tumor tissues. For these experiments, the presence in breast tumor tissues of both CTCF-130 and CTCF-180 was initially confirmed by Western analysis (data not shown). The immunofluorescent staining showed that all tumors inspected were composed predominantly of luminal cells, as they were positive for CK19 and negative for CK14 (a typical example is shown in Fig. 4B). Virtually all tumor cells showed positive staining with the anti-CTCF "poly" and anti-CTCF "mono" antibodies. The heterogeneous character of tumor specimens was evident by the presence of several CK14-positive cells (Fig. 4B, bottom, yellow arrows) and CK19-negative cells (Fig. 4B, top, white arrows). CK14-positive cells displayed morphology of normal myoepithelial cells; most of them were negative for CTCF-130. CK19-negative cells could not be classified in these experiments. Such cells, however, are in a minority in the tumor; therefore, their contribution to the total pool of CTCF in the specimen would be minimal. These observations indicate that the presence of CTCF-130 and CTCF-180 is not restricted to a certain cell type.

**Transition from CTCF-180 to CTCF-130 occurs in the proliferating cultured primary normal breast cells.** The pattern of distribution of CTCF-180 and CTCF-130 suggested that the sole presence of CTCF-180 in normal breast tissues is associated with the quiescent state of the cells, whereas the appearance of CTCF-130 in breast tumors is linked to cell division. If this proposition is correct, CTCF-130 would be expected to appear in dividing cells of primary cultures obtained from the normal breast tissues. To test this hypothesis, primary cultures from normal breast tissues were generated as outlined in Fig. 5A. Using the lysates of cultured primary breast cells, Western blot analysis was done with the anti-CTCF "poly" and anti-CTCF "mono" antibodies to assess for the presence of the CTCF forms. As shown in Fig. 5B, the transition from CTCF-180 to CTCF-130 occurs in normal breast cells after 1 week in culture (the result of a typical experiment is presented). Staining with 5-bromo-2′-deoxyuridine confirmed that these breast cells in culture were proliferating, as a relatively high number of cells were in S phase (Fig. 5C).

![Fig. 4. CTCF-180 and CTCF-130 distribution in normal and tumor breast tissues. Immunofluorescent staining of frozen breast tissue sections from reduction mammoplasty (A) and tumors (B) was done with the anti-CTCF polyclonal ("CTCF poly") and anti-CTCF monoclonal ("CTCF mono") antibodies together with the specific markers of breast luminal (anti-CK19 antibody) and myoepithelial (anti-CK14 antibody) cells. Green, staining with the polyclonal or monoclonal anti-CTCF antibodies; red, staining with the epithelial markers. Nuclei were visualized with 4′,6-diamidino-2-phenylindole staining (blue). Right, merge of the green and red channels. White arrows, CTCF-positive nonluminal cells (B, top); yellow arrows, myoepithelial cells (B, bottom). Images were taken at x40 magnification using the Olympus BX41 microscope.](https://www.aacrjournals.org/doi/figure/10.1158/1078-0432.CCR-09-0329)
Further inspection of primary cultures from normal breast tissues was then carried out using immunofluorescent staining with the antibody specific for CTCF-130 (anti-CTCF “mono”) together with the epithelial markers, CK14, specific for myoepithelial cells, and CK19, specific for luminal cells (Fig. 5D). CTCF-130 was detected in both types of cells, thus supporting the data described in a previous section and Fig. 4.

Appearance of CTCF-180 is correlated with block of cell division. The results described above suggested that CTCF-130 is linked to cell proliferation. To explore whether CTCF-180 may be associated with the quiescent state, we tested if block of the cell cycle could lead to the appearance of CTCF-180. To achieve this, two cell lines, breast cancer cells MCF7 and immortalized noncancer breast cells 226LDM, were arrested in S phase using hydroxyurea alone and hydroxyurea and nocodazole. The flow cytometry analysis confirmed a significant block of the cell cycle at the S and G2-M phases after treatment (Fig. 6A, right). Following inhibition of cell division, an enrichment of the CTCF-180 form occurred in both cell lines, particularly significant in 226LDM-treated cells. This may be explained by the fact that 226LDM cells are derived from normal breast cells originally expressing only CTCF-180; the 226LDM cells may therefore be producing CTCF-180 more efficiently than MCF7 cells.

An arrest of cell division is known to be a characteristic feature that precedes apoptosis, and apoptosis has been shown to be associated with an increased PARP activity (ref. 30, and references therein). We therefore tested whether the appearance of the CTCF-180 form could be detected during the initial phase of apoptosis, by treating MCF7 cells with sodium butyrate, a known inducer of apoptosis (31), for a short period. As shown in Fig. 6B, the characteristic upper form of CTCF (CTCF-180) appeared in MCF7 cells following sodium butyrate treatment (left). The flow cytometry analysis confirmed that a significant proportion of MCF7 cells treated with sodium butyrate for 24 h were arrested in the G2-M phase of cell cycle (Fig. 6B, right). Apoptosis was shown by positive TUNEL staining (Supplementary Fig. 3A) and cleavage of PARP-1 (Fig. 6B) 48 h after treatment. The appearance of CTCF-180 was not restricted to cells treated with sodium butyrate because it was also detected in cells subjected to treatment with other agents causing cell growth arrest (Supplementary Fig. 3B).

Discussion

This study was based on the initial observation of the presence of two different forms of CTCF (slower and faster migrating), with characteristic patterns of distribution, in normal and tumor paired breast tissues. Our experimental evidence confirmed that the slower migrating form of CTCF was a poly-(ADP-ribosyl)ated form of CTCF (CTCF-180), because (a) it migrated identically to the form detected in MCF7 cells, (b) it...
could be immunoprecipitated using anti-PAR antibodies, and (c) it contained peptides specific for CTCF. The sole presence of CTCF-180 band is a characteristic feature of normal breast tissues, whereas both forms, CTCF-180 and CTCF-130, can be simultaneously detected in other normal human tissues. Conversely, CTCF-130, described previously as a non–poly(ADP-ribosyl)ated protein (8), was observed exclusively in breast tumor samples and never in normal breast tissues and therefore was designated as the “breast tumor–specific” form of CTCF. It has yet to be explained why cells in normal breasts produce only CTCF-180; it is possible that this may be linked to a very low proliferation rate in normal breast cells (32). Using Ki-67 as a marker of proliferation (33), we confirmed the absence of Ki-67 in normal breast tissues, whereas high levels of Ki-67 were found in breast tumor tissues (Supplementary Fig. 4). In other normal tissues, Ki-67–positive cells could be detected (Supplementary Fig. 4), although with no clear correlation to the levels of CTCF-130 (Fig. 1C); this is possibly due to the heterogeneous nature of the specimens. Thus, it is conceivable that the appearance of CTCF-130 in other normal tissues may be linked to their proliferation rate. This warrants further investigation.

Identification and characterization of the antibody that specifically recognized only the CTCF-130 form (anti-CTCF “mono”) provided us with a very important tool for more detailed studies using biochemical and imaging techniques. Using this antibody, we found that 87.7% of breast tumors contained CTCF-130. The levels of CTCF-130 varied among tumors, with IRS ranging from 1 to 9. Such a wide variation can be explained by the heterogeneity of breast tumors, which may reflect the different origin and evolution of each tumor (34). From the nine control samples (breast reduction mammoplasty tissue and non-tumor-bearing

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**Fig. 6.** Appearance of CTCF-180 is linked to inhibition of cell division. 
A, CTCF-180 appears in breast cancer cells MCF7 and normal luminal 226LDM cells following treatment with inhibitors of cell cycle. For synchronization in S phase, MCF7 (top) and 226LDM (bottom) cells were treated with hydroxyurea ± thymidine for 48 h; for synchronization in G2-M phase, cells were further incubated for 24 h with nocodazole (see Materials and Methods for details). Concentrations of the chemicals are shown on the diagram. Cell cycle block was confirmed by fluorescence-activated cell sorting analysis (right). Following treatment, cell lysates were produced and resolved by SDS-PAGE and Western blot analysis was done using the polyclonal anti-CTCF antibody (anti-CTCF “poly”; top). The anti-α-tubulin antibody was used as control for protein loading. 
B, CTCF-180 appears in MCF7 cells following treatment with sodium butyrate. MCF7 cells were treated with 5 mmol/L sodium butyrate (NaB) for various lengths of time as indicated on the diagram. Block of cell cycle in G2-M was confirmed by fluorescence-activated cell sorting analysis (data are shown for untreated cells and cells treated for 24 h; right). Lysates obtained from untreated and treated cells were analyzed by Western blot assay using the polyclonal anti-CTCF antibody. The membrane was stripped and subsequently reprobed with the anti-PARP and anti-α-tubulin antibodies.
paired peripheral tissues), eight were negative for CTCF-130 and only one paired peripheral tissue was weakly positive for CTCF-130. This may be due to the influence of the tumor microenvironment; such precedents have been reported previously in the literature (35, 36).

Comparison of the CTCF-130 levels with clinicopathologic parameters revealed a negative correlation between CTCF-130 IRS and tumor size, tumor stage, and positive lymph node status. These three parameters are among traditional predictive/prognostic factors in breast cancer (37, 38). Higher levels of proteins with tumor-promoting features and lower levels of proteins with tumor-suppressing features have been linked to unfavorable prognosis (39–43). In our study, lower levels of CTCF-130 are associated with larger tumors, higher tumor stage, and positive lymph node status, which suggest that the loss of CTCF-130 may be linked to tumor progression. This view harmonizes with the perception of CTCF as a tumor suppressor protein (2, 4, 44, 45). As this is a relatively recent study, we were unable to collect sufficient information to correlate these findings with patient survival and disease recurrence. However, such analysis will be performed in the future.

Our observations imply that (a) the single presence of CTCF-180 is a characteristic feature for normal breast cells, (b) CTCF-130 can be considered as a specific tissue marker for breast tumors, and (c) lower levels of CTCF-130 in tumors can be a marker of unfavorable prognosis. Future studies will examine the utility of these two markers in diagnosis and prognosis of the disease.

Our hypothesis that the unique presence of CTCF-180 [poly (ADP-ribosyl)ated] is associated with the quiescent state of the cells whereas the appearance of CTCF-130 [non-poly(ADP-ribosyl)ated] is a feature of dividing cells was tested using primary breast cultures and cell lines. In these experiments, a link was established between cell division and loss of poly(ADP-ribosyl)ation of CTCF. It should also be noted that the general “pool” of CTCF remained the same: the appearance of CTCF-180 correlated well with the decrease in CTCF-130 and vice versa. This observation supports the hypothesis of the PAR turnover between CTCF-130 and CTCF-180 regulated by the PARPs-poly(ADP-ribose) glycohydrolase enzymatic pathways. The PAR homeostasis resulting from the appropriate balance between PARP and poly(ADP-ribose) glycohydrolase activities is vital for normal cell function; the disturbance of PAR homeostasis leads to dramatic changes in the cells and may result in disease development (46). In our case, the poly(ADP-ribosyl)ation status of CTCF, which is likely to define CTCF function, may generally depend on the state of cell metabolism, which in turn is related to the state of the cell (quiescent, proliferating).

It has recently been reported that poly(ADP-ribosyl)ation is necessary for transition from quiescence to proliferation at least for some cell types (fibroblasts and monocytes; 47). Although this observation seems to contradict our findings, the discrepancy can be explained by the existence of CTCF-specific poly (ADP-ribosyl)ation pathways. Such pathways may rely on direct interaction between PARP-1 and CTCF, the ability of CTCF to directly activate PARP-1 poly(ADP-ribosyl)ation, and a feedback poly(ADP-ribosyl)ation of CTCF (15). We hypothesize that extracellular and intracellular stimuli may specifically affect the activity of CTCF, which in turn leads to the activation of the “CTCF-PARP-1 circuit,” independently of other poly(ADP-ribosyl)ation events in the cell. The tight feedback control between poly(ADP-ribosyl)ation and de-poly(ADP-ribosyl)ation of CTCF may be characteristic for normal cells, whereas the combined presence of CTCF-130 and CTCF-180 in cell lines and tumors may reflect de-regulation of the “CTCF-PARP-1” feedback loop.

Several additional issues arise from this study. First, what molecular mechanisms are behind the actions of CTCF-130 and CTCF-180? Do these forms control different CTCF target genes in normal and tumor breast tissues? If this is the case, then it is possible that the “ unplanned” appearance of CTCF-130 may lead to competition of CTCF-130 with CTCF-180 and functional deregulation of the CTCF-180 DNA targets. This scenario implies that CTCF-130 and CTCF-180 “coexist” in the same cells. In support of this observation is the presence of significant amounts of CTCF-180 in tumors. This cannot simply be explained as derived from a small number of normal cells as the tissues were prepared by an experienced pathologist and verified as composed of mainly tumor cells. However, the simultaneous presence of the two forms is impossible to formally establish in the absence of an antibody specifically recognizing CTCF-180.

One of the manifestations of the simultaneous presence of CTCF-130 and CTCF-180 is the elevation of total levels of CTCF in breast tumor cells. In breast tumor cell lines, the increased CTCF levels, compared with normal breast cells, are attributed to higher levels of CTCF-130 (7). Our previous reports linked higher levels of CTCF in breast cell lines to resistance of these cells to apoptosis; the relevance of these findings to primary breast tumors still needs to be confirmed, as it is not known how this function of CTCF may be modulated by the presence of CTCF-180 (7). This observation raises the issue of adequacy of cancer cell lines in breast cancer research, widely debated in the literature (48). It is especially related to diverse evolution of tumor cells in vivo and in vitro when different selective pressures on tumor cells are applied in different environments.

The situation is further complicated due to aberrant expression of BORIS (or CTCF-L), a parologue of CTCF, in breast tumors (23). The simultaneous presence of BORIS and CTCF-130 in breast tumors indeed has been observed (data not shown), although it is not known which protein appears first. Negative regulation of BORIS by CTCF in normal cells has been reported previously (49). It is tempting to speculate that, in the process of tumor development, this function may alter due to the changes in the CTCF poly(ADP-ribosyl)ation status; this proposition will require further investigation. It is also puzzling why higher levels of CTCF-130, a marker for proliferation, are associated with smaller tumor size and decreased stage. We hypothesize that this phenomenon may be associated with possible change of function of CTCF-130 in the process of tumor evolution. In this context, a complex interplay between CTCF-130, CTCF-180, and BORIS also needs to be considered. These possibilities deserve further investigation.

Profiling the genes controlled by CTCF-180 and CTCF-130 in normal and tumor tissues will certainly help us understand which biochemical and genetic pathways are governed by these proteins in the normal and transformed breast cells and will pave the way to understanding the functional role of these forms of CTCF.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Acknowledgments

We thank C. Chandrasekharan, P. Murray, K. Roeke, and K. Reeven for help with tissue collection and information; I. Seddon for specialist advice and expertise on breast tumor tissues; F. Burri and J. Benhattar for providing human tissues from the Tissue Bank at the Institute of Pathology (Lausanne, Switzerland); D. Edwards and C. Pennington for sharing technologies and helpful discussions; E. Lane for the mouse anti-Ck19 for help with imaging; and I. Morrison for help with fluorescence-activated cell sorting, imaging, and data analysis.

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