Loss of Cyclin D2 Expression in the Majority of Breast Cancers Is Associated with Promoter Hypermethylation

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ABSTRACT

Cyclin D2 is a member of the D-type cyclins, implicated in cell cycle regulation, differentiation, and malignant transformation. It was noted previously that cyclin D2 is not expressed in the majority of breast cancer cell lines, whereas abundant expression was detected in finite life span human mammary epithelial cells. By reverse transcription-PCR and Western blot analysis, we extended this finding to primary breast carcinomas and showed that the majority of these tumors lack expression of cyclin D2 mRNA (18 of 24) and protein (10 of 13). In contrast, both luminal and myoepithelial subpopulations of normal breast tissues expressed cyclin D2. Hypermethylation of the Cpg island in the promoter was detected by methylation-specific PCR in nearly half of the breast cancers (49 of 106) and was associated with silencing of cyclin D2 gene expression. Promoter hypermethylation was also detected in ductal carcinoma in situ, suggesting that loss of cyclin D2 expression is an early event in tumorigenesis. Our results suggest that loss of cyclin D2 expression is associated with the evolution of breast cancer.

INTRODUCTION

In mammalian cells, replication is regulated in an orderly fashion from G1 to S to mitosis by phase-specific oscillations in the level of cyclins, cdks, and cdk inhibitors. The D-type cyclins (cyclins D1, D2, and D3) are involved in regulation of transition from G1 to S during the cell cycle. Their critical function is to activate cdk4 and cdk6, leading to the phosphorylation of RB, the retinoblastoma tumor suppressor protein. This, in turn, leads to release of transcription factors such as E2F from RB-mediated repression, which then activate transcription of genes involved in DNA synthesis and thus trigger the onset of S-phase (reviewed in Refs. 1–3).

Given the critical role of the D-type cyclins in cell cycle regulation, their abnormal or untimely expression could disrupt the cell cycle and therefore render them growth-promoting genes or oncogenes. It has been indicated that cyclin D1 is a putative proto-oncogene and is overexpressed in a number of tumor types including lymphomas, breast cancer, and thyroid carcinoma (1). Aberrant expression of cyclin D2 has been noted in human ovarian granulosa cell tumors and testicular germ cell tumor cell lines (4). Recently, evidence was presented suggesting that cyclin D2 is a direct target of Myc, and that accumulation of cyclin D2 contributes to sequestration of the cell cycle inhibitor p27 and to cell cycle entry (5, 6). A recent analysis of myc-induced genes using oligonucleotide microarrays also revealed cyclin D2 as a target of c-myc in primary human fibroblasts (7).

Furthermore, in B-lymphocytic tumors such as Burkitt’s lymphoma, transcriptional activation by chromosomal translocation renders c-myc a potent inducer of cell proliferation by promoting the expression of cell cycle activators such as cyclin D2, cyclin E, and cdk4 (8).

Although well known for their proliferation-promoting activity, the D-type cyclins were also shown to have growth-inhibitory effects, based on their ability to induce a senescence-like phenotype (reviewed in Ref. 9) and to inhibit cell proliferation (10). In fact, Cyclin D2 was unique among the three D-type cyclins, being up-regulated manifold under conditions of growth arrest in phenotypically normal human and murine fibroblasts. Furthermore, ectopic overexpression of cyclin D2 effectively blocked cell cycle progression, suggesting an alternate role for cyclin D2 in promoting exit from the cell cycle and maintaining a nonproliferative state. The fact that high levels of cyclin D2 are expressed in normal human tissues composed primarily of nonproliferating contact-inhibited cells such as breast and brain (10) is consistent with those in vitro observations.

At least four previous studies have demonstrated that cyclin D2 mRNA and protein were absent in almost all breast cancer cell lines examined, whereas cultured normal breast epithelial cells had abundant expression (11–14). Furthermore, our serial analysis of gene expression and array analysis revealed low or absent cyclin D2 mRNA expression in primary breast cancers as compared with finite life span HMECs (15), again suggesting that cyclin D2 expression is incompatible with proliferation in mammary epithelial cells. The significance of the differential expression pattern in HMECs compared with breast cancer cell lines was questioned by a report stating that cyclin D2 is expressed in the myoepithelial cells that form the outer lining of the mammary ducts but not in the luminal epithelial cells (13). This distribution could explain the apparent tumor-specific loss of cyclin D2, because most carcinomas arise from the luminal cells (13). We have now reinvestigated the expression of cyclin D2 in these two cell types from the normal mammary gland and found that cyclin D2 mRNA is expressed in both luminal and myoepithelial cells. We also demonstrated lack of cyclin D2 mRNA and protein in the majority of breast carcinomas and showed that silencing of gene expression correlated with hypermethylation of the cyclin D2 promoter.

MATERIALS AND METHODS

Cell Lines and Tissues. The breast cancer cell lines MDA-MB435, MCF7, T47D, SKBR3, ZR75.1, MDA-MB468, HS578T, and MDA-MB231 and the immortal and nontumorigenic HMECs MCF10A and HBL100 (contains SV40 virus genome) were obtained and maintained in culture according to instructions (American Type Culture Collection, Rockville, MD). The two tumor cell lines, 21PT, derived from a primary tumor, and 21MT, from the metastasis of the same patient, were propagated as described (16). HMEC H16N (immortalized with human papillomavirus) were kindly provided by Dr. Vimla Band (Tufts University, Boston, MA). Cultured finite life span human breast epithelial cell strains 04372, 9F1403, and 166372 were purchased from Clonetics (Walkersville, MD), and HMEC strains 1–26, 11–24, and 3–14 were kindly provided by Dr. Steve Ethier (University of Michigan, Ann Arbor, MI). Finite life span HMEC 184 and the immortalized HMECs 184/1 (passage 15 and 99) and 184B5 were kindly provided by Dr. Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA).
National Laboratory, Berkeley, CA) and grown as described.\(^4\) Cell extracts from finite life span HMECs 70N and 81N were kindly provided by Dr. Khandan Keyomarsi (SUNY Albany, Albany, NY). Mammary organoids were prepared from reduction mammoplasty specimens of women with benign or no abnormalities in the breast after collagenase digestion as described (17). Human mammary luminal and myoepithelial cells were prepared by progressive collagenase digestion of breast tissue, sedimented to obtain organoids (ductal and lobulo-alveolar fragments), cultured short term, and finally highly enriched by using an immunomagnetic separation technique (18, 19).

Primary breast tumor tissues were obtained after surgical resection at the Johns Hopkins University and Duke University, and stored frozen at \(-80^\circ C\). Samples containing \(>50\)% tumor cells were selected after microscopic examination of representative tissue sections from each tumor. Microdissection of carcinoma and DCIS lesions was performed on 8-\(\mu\)m cryosections using a laser capture microscope (20) or by manually scraping the cells with a 25-gauge needle under \(\times 40\) magnification (21). Genomic DNA was extracted by incubating the microdissected cells at 55\(^\circ C\) for 12 h in 50 \(\mu\)l of buffer containing 10 \(\mu\)M Tris Cl (pH 8.0), 1 \(\mu\)M EDTA, 0.1\% Tween 20, and 0.5 \(\mu\)g/\(\mu\)l proteinase K. The extract was heat inactivated at 85\(^\circ C\) for 5 min and used directly for southern bisulfite treatment (22).

**MSP.** One \(\mu\)g of genomic DNA or the 50-\(\mu\)l extract of microdissected cells (as described above) was treated with sodium bisulfite as described by Herman et al. (22) and was analyzed by MSP using primer sets located within the CpG-rich island in the cyclin D2 promoter. Primers specific for unmethylated DNA were 5'-GTTAGTTGATTTTGTTGATG-3' (sense, \(-1616\) to \(-1594\)) and 5'-TAAAAATCCACCAACAACTCA-3' (antisense, \(-1394\) to \(-1414\)) and yielded a 222-bp PCR product. Primers specific for methylated DNA were 5'-TACGTTAGGTAGGCTAGC-3' (sense, \(-1427\) to \(-1409\)) and 5'-CGAAATATCTACGTAACGG-3' (antisense, \(-1152\) to \(-1171\)) and yielded a 276-bp PCR product. The PCR conditions were as follows: 1 cycle of 95\(^\circ C\) for 5 min “hot start,” then addition of 1 \(\mu\)l of Taq polymerase (RedTaq; Sigma); 35 cycles of 95\(^\circ C\) for 30 s, 55\(^\circ C\) for 30 s, and 72\(^\circ C\) for 45 s; and 1 cycle of 72\(^\circ C\) for 5 min. The PCR products were resolved by electrophoresis in a 2% agarose gel.

**Treatment of Cells with 5-aza-dc and TSA.** Cells were seeded at a density of 1 x 10\(^5\) cells/100-mm plate. Twenty-four hour later, cells were treated with 0.75 \(\mu\)M 5-aza-dc (Sigma) or with 100 ng/ml of TSA (Sigma; Refs. 23 and 24). Total cellular DNA and RNA were isolated at 0, 3, and 5 days after addition of 5-aza-dc and at 0, 24, and 48 h after addition of TSA, as described above.

**RT-PCR.** Total RNA was treated with RNase-free DNase (Boehringer-Mannheim; 0.5–1 units/\(\mu\)l) at 37\(^\circ C\), followed by heat inactivation at 65\(^\circ C\) for 10 min. Reverse transcription reactions contained 2 \(\mu\)g of DNase-treated RNA, 0.25 \(\mu\)g/\(\mu\)l pdN6 random primers (Pharmacia), 1 \(\times\) first-strand buffer (Life Technologies, Inc.), 1 \(\times\) dNTP, and 200 units of Moloney murine leukemia virus-RT (Life Technologies, Inc.), and were incubated for 1 h at 37\(^\circ C\), followed by heat inactivation at 75\(^\circ C\) for 5 min. PCR was performed using the primers 5'-CAGGAGGTCGCTTGGCAGC-3' (sense) and 5'-GCGACCTAATGCCCCAG-3' (antisense) for cyclin D2 and primers 5'-AGCCATGGACACGACTGTC-3' (sense) and 5'-GCAGGCACGACGACG-3' (antisense) for cyclin D1. Coamplified products of 36B4, a “housekeeping” ribosomal protein gene, were used as an internal control, using primers 5'-GATTGGCTAC-3' (sense) and 5'-CAGGGCCGAGACGACAAAGGC-3' (antisense). The 25-\(\mu\)l reactions contained 1 \(\times\) buffer (2 \(\times\) Reaction Mix; BRL) and 100 \(\times\) of each primer. The PCR conditions were: 1 cycle of 94\(^\circ C\) for 1 min “hot start,” then addition of 1 \(\times\) of Taq polymerase (RedTaq; Sigma); 1 cycle of 94\(^\circ C\) for 2 min; 35 cycles of 94\(^\circ C\) for 15 s, 55\(^\circ C\) for 30 s, 72\(^\circ C\) for 45 s, and finally, 72\(^\circ C\) for 5 min. The PCR samples were resolved by electrophoresis on a 2% agarose gel.

**Western Blot Analysis.** Proteins were extracted from cell pellets and from 8-\(\mu\)m cryosections of primary breast tumors in buffer containing 20 \(\mu\)M Tris (pH 7.5), 150 \(\mu\)M NaCl, and phenylmethylsulfonyl fluoride (100 \(\mu\)g/ml), and sonicated. Ten \(\mu\)g of protein was fractionated on 12.5-\% SDS-PAGE and transferred by electrophoresis to a nylon membrane. The blot was incubated with anti-cyclin D2 antibody (Ab-4, “cocktail” mouse monoclonal antibodies; Neomarkers, San Diego, CA) diluted 1:200 in 5% skim milk for 2 h at room temperature. Horseradish peroxidase-conjugated antibody antimouse IgG (Amersham) was used at 1:1000, and binding was revealed using enhanced chemiluminescence (Amersham).

**RESULTS**

Cyclin D2 mRNA Expression Is Lost in Breast Cancer. Serial analysis of gene expression and subsequent microarray analysis had previously revealed that, compared with finite life span HMECs, cyclin D2 expression was significantly lower in a small panel of primary breast tumors (15). To determine the validity of these findings, we investigated expression of cyclin D2 by RT-PCR in three finite life span and six immortal HMECs, 11 breast cancer cell lines, and 24 primary breast carcinomas. A ribosomal protein RNA, 36B4, was coamplified as an internal control. Abundant expression of cyclin D2 mRNA was noted in all three finite life span HMECs and in four of six immortalized HMECs (Fig. 1A; Table 1). The two immortalized HMECs lines lacking cyclin D2 expression were HBL100 and MCF10A (data not shown; Table 1). In contrast, 10 of 11 breast cancer cell lines showed no detectable expression of cyclin D2. Only one breast cancer cell line, HS578T, expressed a low but detectable level of cyclin D2 (Fig. 1A; Table 1). Likewise, the results with primary tumors reflected the findings in cultured cells. Eighteen of 24 primary breast carcinomas expressed significantly lower levels of cyclin D2 mRNA (Fig. 1B and data not shown) as compared with finite life span HMEC 184 and five other HMECs (Fig. 1A; Table 1). As an additional control for cyclin D2 expression, we analyzed the expression of cyclin D1 in the same panels of cell lines and tumors. Consistent with the observations of others (11–14, 25, 26), cyclin D1 mRNA was detectable in all of the cell lines and primary breast tumors tested (Fig. 1). Thus, in both breast cancer cell lines and primary tumors, specific loss of cyclin D2 mRNA expression but not of cyclin D1 expression was observed. These results extend and confirm our data (15) and others (11, 13, 14, 25, 27).

**Western Analysis Reveals Loss of Cyclin D2 Protein in Primary Tumors.** For Western blot analysis, we used specific anti-cyclin D2 antibodies that did not cross-react with cyclin D1. Although cyclin D2 protein was clearly detected in all seven HMECs tested (11–24, 1–26, 208, and 212), cyclin D2 protein was absent in all 11 primary breast tumor samples analyzed by RT-PCR (Fig. 1B). Coamplified products of 36B4, a “housekeeping” ribosomal protein gene, were used as an internal control, using primers 5'-GATTGGCTAC-3' (sense) and 5'-CAGGGCCGAGACGACAAAGGC-3' (antisense). The 25-\(\mu\)l reactions contained 1 \(\times\) buffer (2 \(\times\) Reaction Mix; BRL) and 100 \(\times\) of each primer. The PCR conditions were: 1 cycle of 94\(^\circ C\) for 1 min “hot start,” then addition of 1 \(\times\) of Taq polymerase (RedTaq; Sigma); 1 cycle of 94\(^\circ C\) for 2 min; 35 cycles of 94\(^\circ C\) for 15 s, 55\(^\circ C\) for 30 s, 72\(^\circ C\) for 45 s, and finally, 72\(^\circ C\) for 5 min. The PCR samples were resolved by electrophoresis on a 2% agarose gel.

Both Luminal and Myoepithelial Cells of the Breast Express Cyclin D2. It has been reported previously that cyclin D2 is expressed in myoepithelial but not in luminal epithelial cells of the breast (13). If this were accurate, then lack of expression of cyclin D2 in breast cancers would be expected, because the vast majority of these tumors originate from luminal rather than myoepithelial cells. This conclusion was based, however, on results from a single HMEC preparation (13). We have reinvestigated this question with a larger panel of tissues. Luminal and myoepithelial cells isolated from four normal mammaryplasty specimens from women of ages 18–33 were used. Paired luminal and myoepithelial cells were obtained from the same mammaryplasty specimens of two women. Each cell type was purified using immunomagnetic beads. The human luminal and myoepithelial cells were separated by virtue of their exclusive expression of epithelial membrane antigen and common acute lymphoblastic leukemia antigen, respectively. The purity of the populations was checked by immunocytochemistry using cytokeratins 8 and 19 as markers for luminal cells and cytokeratin 14 as a marker for myoepithelial cells. These tests showed that the final population was 95–99% pure in each case (18, 19). We then tested for cyclin D2 expression in the purified cell preparations by RT-PCR. We observed cyclin D2 expression in four of four purified luminal epithelial and also in four of four myoepithelial cell preparations (Fig. 3). However, one luminal epithelial cell sample expressed a noticeably lower level of cyclin D2 (Fig. 3, Lane 3) compared with the other three. Additionally, another finite life span HMEC, 184, also stained for luminal cell markers, cytokeratins 8 and 18 and mucin, but not for the myoepithelial cell marker cytokeratin 14. As seen in Fig. 1A, HMEC 184 and its three derivatives also expressed cyclin D2 mRNA (Fig. 1A). Thus, cyclin D2 mRNA was expressed in all five of five luminal and four of four myoepithelial cell preparations from the normal breast. Immunohistochemical analysis of normal breast tissue using anti-cyclin D2 antibodies (Neomarkers) was attempted but was unsuccessful, because nonspecific staining was observed.

The Cyclin D2 Promoter Is Hypermethylated in Breast Cancer Cell Lines and Primary Tumors. In somatic cells, ~80% of the CpGs are methylated. Exceptions to this are the CpG islands in the promoter region of many genes. CpG islands are CG-rich regions of DNA, ~1 kb in length, present in the promoters of >60% of human genes. Normally, CpG islands are unmethylated, and the chromatin in those sites is enriched in hyperacetylated histone and deficient in histone H1, characteristics of active chromatin. Both unmethylated and methylated DNA are assembled into nucleosomes (reviewed in Refs. 28 and 29).

The cyclin D2 promoter contains a CpG-rich region at 1000–1600 bp 5′ to the translation start site (Fig. 4A). To test whether loss of cyclin D2 expression in breast cancer is associated with aberrant methylation, we designed primers for MSP to rapidly screen for cyclin D2 promoter methylation. Hypermethylation at the CpG rich region was detected in 11 of 11 breast cancer cell lines (data not shown; Table 1) that lacked expression of cyclin D2 mRNA (Fig. 1A) and protein (11, 13, 14, 25, 27). Hypermethylation was also noted in 49 of 106 (46%) primary breast carcinomas (Table 1; Fig. 4B; data not shown).

To determine whether cyclin D2 promoter-methylation is a tumorspecific phenomenon, DNA from histopathologically normal breast tissues adjacent to the surgically resected cancer were tested. All 11 samples of normal breast epithelial tissue adjacent to carcinoma were unmethylated at the CpG sites tested by MSP (Fig. 4C; Table 1).

To further confirm that cyclin D2 hypermethylation is confined to tumors and does not occur in the normal breast, we examined normal mammary epithelial cells prepared by a variety of techniques. By

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Table 1  Cyclin D2 methylation correlates with loss of mRNA and protein expression in breast cancer

<table>
<thead>
<tr>
<th>Tissue/cells</th>
<th>Expression</th>
<th>No. with methylated cyclin D2/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral blood cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMECs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finite life span</td>
<td>184, 166372, 70, 81N, 04372</td>
<td>3/3</td>
</tr>
<tr>
<td>Immortal</td>
<td>184A1–15, 184A1–99, H16N, 184B5</td>
<td>4/4</td>
</tr>
<tr>
<td>HBL100, MCF10A</td>
<td>0/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Fractionated cells</td>
<td>Luminal</td>
<td>6/6</td>
</tr>
<tr>
<td>Myoepithelial</td>
<td>6/6</td>
<td>4/4</td>
</tr>
<tr>
<td>Mammary organoids</td>
<td>Prep 1</td>
<td>6/6</td>
</tr>
<tr>
<td>Prep 2</td>
<td>3/3</td>
<td>0/7</td>
</tr>
<tr>
<td>Normal breast margin adjacent to tumor</td>
<td></td>
<td>0/1</td>
</tr>
<tr>
<td>Subtotal for HMECs</td>
<td>21/23 (91%)</td>
<td>11/11 (100%)</td>
</tr>
<tr>
<td>70N, 166372, 81N, 9F1403, and 184A1</td>
<td>0/2</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>49/106 (46%)</td>
<td></td>
</tr>
<tr>
<td>70N, 166372, 81N, 9F1403, and 184A1</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>11/11</td>
<td></td>
</tr>
<tr>
<td>Primary tumor/adjacent normal</td>
<td>3/13</td>
<td>8/13</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>3/13</td>
<td>8/13</td>
</tr>
<tr>
<td>Primary tumor/adjacent normal</td>
<td>3/11</td>
<td>6/11</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>3/11</td>
<td>6/11</td>
</tr>
<tr>
<td>Primary tumor/adjacent normal</td>
<td>3/11</td>
<td>6/11</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>3/11</td>
<td>6/11</td>
</tr>
<tr>
<td>Subtotal for primary carcinomas</td>
<td>6/24 (25%)</td>
<td>3/24 (13%</td>
</tr>
<tr>
<td>Luminal and Myoepithelial Cell Markers</td>
<td>49/106 (46%)</td>
<td></td>
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</tbody>
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70N, 166372, 81N, 9F1403, and 184A1, was undetectable in the majority (10 of 13) of primary breast carcinomas (Fig. 2; Table 1). Of note, primary breast carcinomas that expressed very low or undetectable levels of cyclin D2 mRNA (nos. 03, 11, 30, 38, 40, 63, and 70 in Fig. 1B) showed no detectable cyclin D2 protein (Fig. 2). HMECs that were derived from normal breast tissue and expressed high levels of cyclin D2 mRNA (Fig. 1A) showed clearly detectable levels of cyclin D2 protein as well (Fig. 2).

Fig. 2. Western blotting analysis of cyclin D2 expression in breast. Lysates (10 μg of protein) from finite life span HMECs 11–24, 1–26, 70N, 166372, 81N, 9F1403, and immortalized HMEC 184A1, as well as 13 primary breast carcinomas were immuno-blotted with a cyclin D2 mouse monoclonal antibody mixture as described in “Materials and Methods.” C., carcinoma.

Fig. 3. Cyclin D2 (Cyc D2) expression in breast luminal and myoepithelial cell populations purified by an immunomagnetic separation technique (18, 19). Lane M, size markers; Luminal and myoepithelial samples 1 and 2 were pairs derived from two individuals, whereas luminal samples 3 and 4 and myoepithelial samples 3 and 4 were each obtained from a different individual. Complified 36B4 RT-PCR product serves as an internal loading control.
MSP analysis, cyclin D2 promoter was found to be unmethylated in seven mammary organoid preparations from reduction mammaplasties and in four HMECs cultured from nonmalignant breasts (Fig. 4D).

The only exception to this finding was in immortalized HMECs HBL100 and MCF10A, which contained hypermethylated cyclin D2. As expected, these HMECs were the only two that did not express cyclin D2 mRNA (Table 1; data not shown).

To rule out the contribution of inflammatory blood cells present in breast cancer specimens as the source of methylated cyclin D2, we tested 10 samples of peripheral blood lymphocytes from noncancer patients. All 10 peripheral blood lymphocytes contained unmethylated cyclin D2 alleles (Fig. 4E).

As shown in Fig. 2, expression of cyclin D2 protein was undetectable in 10 of the 13 primary breast cancers tested (tumor panel 1; Table 1). By MSP analysis, hypermethylation of the cyclin D2 promoter was observed in 8 of 13 primary breast carcinomas (data not shown). In seven of eight tumors (nos. 30, 38, 40, 63, 70, 17, and 72), methylation of cyclin D2 correlated with lack of cyclin D2 protein. In the one exception (tumor 36), both mRNA and protein expression was seen, despite the presence of a hypermethylated cyclin D2 gene. Five of 13 tumors contained unmethylated cyclin D2 sequences. Among these, two (tumor nos. 13 and 35) showed expression of mRNA and protein, whereas three (tumor nos. 03, 11, and 21) did not. Thus, 87% of the tumors showed a correlation between hypermethylation of the cyclin D2 promoter and silencing of gene expression. The observation that some tumors lacked protein expression despite the unmethylated status of the gene suggests that although methylation may constitute a major pathway for silencing of cyclin D2 expression in breast cancers, alternative pathways account for the loss of the protein in a proportion of these tumors.

Cyclin D2 Promoter Hypermethylation in Preneoplasia. DCIS is a preneoplastic lesion with a potential for progression to invasive cancer. To determine whether hypermethylation of the cyclin D2 promoter occurs early in the evolution of breast cancer, we performed MSP analysis on samples from 13 primary breast tumors that contained both invasive and noninvasive components. One or more areas of carcinoma (n = 17) and adjacent DCIS (n = 18) were carefully microdissected from the same section. Hypermethylated cyclin D2 was present in 8 of 17 carcinomas and in 8 of 18 DCIS samples (Table 2; Fig. 4F; data not shown). In all of the 13 cases, the methylation status of both carcinoma and DCIS lesions from the same tumor was concordant. This finding suggests that alteration of cyclin D2 expression is an early event and may precede transformation to the fully malignant stage of invasive carcinoma.

Reexpression of Cyclin D2 mRNA in Breast Cancer Cell Lines. Breast cancer cell lines MDA-MB231 and MCF7 do not express cyclin D2 mRNA (Fig. 1A) or protein (11–13). If silencing of expression was mediated by promoter methylation and/or altered chromatin conformation, then demethylation of the gene by exposure to 5aza-dC or treatment with the histone deacetylase inhibitor TSA should result in reexpression of the gene. Indeed, when MDA-MB231 and MCF7 cells were exposed to 5-aza-dC in culture, the cyclin D2 promoter was partially demethylated (as analyzed by MSP), and cyclin D2 mRNA expression was restored (as analyzed by RT-PCR; Fig. 5A). Although the unmethylated cyclin D2 band is visible by day 3 of treatment with 5-aza-dC, the mRNA was seen only at the day 5 time point (Fig. 5A). There was a lag period between demethylation of the promoter sequences and mRNA expression of cyclin D2 (Fig. 5A), indicating that demethylation of the gene precedes gene expression. Furthermore, exposure to TSA also led to reexpression of the cyclin D2 mRNA (Fig. 5B), but no change in the methylation status of the gene was observed (data not shown). These results suggest that methylation and histone deacetylation at the

### Table 2 Incidence of methylated cyclin D2 in microdissected DCIS

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. microdissected</th>
<th>No. with unmethylated cyclin D2</th>
<th>No. with methylated cyclin D2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast carcinoma</td>
<td>17</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>DCIS</td>
<td>18</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

* A total of 13 primary invasive ductal carcinomas (not otherwise specified) were microdissected.

![Fig. 5. Treatment with 5-aza-dC (5-Aza-dC) or TSA restored expression of cyclin D2 (Cyc D2) in breast cancer cells. MDA MB231 cells were treated with 5-aza-dC or TSA as described in "Materials and Methods." DNA and RNA were extracted from cells harvested at the indicated times after exposure to the chemicals. A, the top panel is the MSP analysis of the cyclin D2 promoter. The lower panel is a RT-PCR analysis of the same cells treated with 5-aza-dC, showing reexpression of cyclin D2 by 5 days. U, unmethylated; M, methylated. B, RT-PCR of cyclin D2 in cells after treatment with TSA. Note reexpression of cyclin D2 mRNA after 24 h. There was no change in the methylation status in the cells treated with TSA (data not shown).](cancerres.aacrjournals.org)
promoter region may play a functional role in suppressing the expression of cyclin D2 in breast cancer.

**DISCUSSION**

The cyclin D1–3 proteins are involved in regulation of the cell cycle through phosphorylation and inactivation of the retinoblastoma protein and activation of cyclin E, leading to transition of the cells from G1 to DNA synthesis. In addition to their role in cell cycle regulation, the D-type cyclins have been implicated in differentiation and neoplastic transformation. Overexpression of cyclin D2 has been reported in gastric cancer and was shown to correlate with disease progression and poor prognosis (30, 31). Overexpression of cyclin D2 was also noted in ovarian granulosa cell tumors and testicular germ cell tumor cell lines (4). In the case of the breast, higher levels of cyclin D1 were noted in 30–50% of the tumors when compared with normal breast epithelium; this increased expression was ascribed to gene amplification as well as accumulation of the protein (3, 25, 32). The roles of cyclin D2 and D3 in breast cancer were less thoroughly investigated. Courjal et al. analyzed mRNA expression of all three cyclins in 132 breast tumors. They reported that cyclin D1 was overexpressed in 19% of the tumors, but cyclin D2 or D3 overexpression was never observed (25). Furthermore, several groups have reported the reduced or lack of expression of cyclin D2 in breast cancer cell lines (11–14). In this report, we extend this observation to primary breast cancers and show diminished cyclin D2 mRNA and lack of protein expression in the majority of breast cancers tested. These findings may indicate that cyclin D2 is involved in a vital tumor suppressor function in normal breast tissue, and that its loss may be related to tumorigenesis.

It has been suggested previously that cyclin D2 is expressed in myoepithelial but not in luminal epithelial cells of the breast (13). If this were the case, lack of expression of cyclin D2 in breast cancers would be anticipated, because the vast majority of these tumors originate from luminal rather than myoepithelial cells. However, by RT-PCR, we detected cyclin D2 expression in four mammary luminal epithelial cultures of the 184 series (Fig. 1A) and in all four of four purified luminal epithelial cell extracts (Fig. 3), as well as in four of four myoepithelial cell extracts (Fig. 3). Therefore, we believe that the gene is expressed in both of these cell subtypes. One could attribute the presence of cyclin D2 RT-PCR product to the high sensitivity of the assay in amplifying even a few myoepithelial cells contaminating the luminal cell preparations. Our Western analysis of multiple HMEC samples (including the 184 series) counters this argument, because this less-sensitive technique detected cyclin D2 protein in all seven samples tested (Fig. 2). Of note, of four fractionated luminal cell preparations, one expressed a lower level of cyclin D2 mRNA (Fig. 3, sample 3), in concordance with the previous report. It should be pointed out that the published report was based on the findings in one luminal cell preparation (13). Possibly, cyclin D2 is downregulated under certain circumstances, such as stress, hormonal fluctuations, or culture conditions. Much larger panels of fractionated cells need to be examined, and further research is warranted to explore the impact of physiological influences on cyclin D2 levels in normal mammary cells.

Searching for a mechanism underlying the consistent loss of cyclin D2 expression in breast cancers, we tested the cyclin D2 promoter for CpG hypermethylation as a possible cause of gene silencing. CpG hypermethylation is an epigenetic, heritable change that appears to be tightly associated with the formation of repressive chromatin (28, 29). By MSP analysis, cyclin D2 promoter hypermethylation was detected in 49 of 106 (46%) of the tumors.

The strength of the correlation between hypermethylation of the promoter and silencing of gene expression would provide an assessment of the importance of DNA methylation in cyclin D2 gene regulation. Methylation of cyclin D2 promoter, mRNA, and protein expression were studied in a panel of 13 primary breast carcinomas. In 7 tumors, hypermethylation of the gene correlated with low or no cyclin D2 mRNA and lack of protein expression (Figs. 1B and 2). The one exception to this finding was tumor 36, which showed expression of mRNA and protein but contained hypermethylated cyclin D2. This observation of lack of correlation between hyper-methylation and expression, albeit in one tumor, has precedence in the published literature (23, 24, 33, 34). In this tumor, competing influences of transcription complexes with histone acetylase and histone deacetylase activities appear to have favored the former, allowing gene transcription to occur despite promoter methylation. Interestingly, treatment of breast cancer cells, MDA-MB231, with TSA, a histone deacetylase inhibitor, resulted in reexpression of the cyclin D2 mRNA within 24 h with no change in the methylation status (Fig. 5B). Altogether, these findings indicate that an appropriate conformation of the chromatin in which the cyclin D2 promoter is embedded is sufficient for transcription and can override the effects of DNA hypermethylation.

The results of this study show that in breast cancers, cyclin D2 protein expression is undetectable in 80% of the tumors, and that silencing of cyclin D2 gene expression may be attributed to tumor-specific methylation in ~50% of the tumors tested. In fact, methylation was proposed previously as the mechanism suppressing cyclin D2 expression in resting primary B lymphocytes. In that instance, infection of these cultured cells with EBV led to the demethylation and reexpression of the cyclin D2 gene (35). Other repressive mechanisms likely contribute to silencing of the gene in the remaining 30% of cyclin D2-nonexpressing tumors. The high rate of aberrant methylation in DCIS (44%) also suggests that loss of cyclin D2 may be an early event in the malignant transformation of breast cancers.

The frequency of cyclin D2 promoter hypermethylation in breast cancer and its absence in normal breast tissue and blood cells, as shown in this report, make it an excellent candidate marker for breast malignancy. On the basis of the high sensitivity of PCR-based assays for detection of methylated genes, cyclin D2 may prove to be useful for detection of cancer in minute samples such as ductal fluid, fine needle biopsies, pathologically negative sentinel lymph nodes, and blood from metastatic breast cancer patients. We are currently exploring the applications of methylated alleles of cyclin D2 as a tumor marker.

Finally, the fact that cyclin D2 expression is lost in breast tumors strongly suggests that the function of this protein is not limited to its well-known role in G1-S transition during the cell cycle. Breast cancer-specific loss of cyclin D2 expression, confirmed by the present study, sets the stage for investigation of a possible role for cyclin D2 in the terminal differentiation and senescence of human breast epithelial cells and exploration of the part its loss may play in the evolution of breast cancer.

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Loss of Cyclin D2 Expression in the Majority of Breast Cancers Is Associated with Promoter Hypermethylation

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