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Report

Cyclin D1 overexpression in a model of human breast premalignancy: preferential stimulation of anchorage-independent but not anchorage-dependent growth is associated with increased cdk2 activity

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Key words: cyclin D1, premalignant disease progression, anchorage-independent growth, MCF-10A cells

Summary

Cyclin Dl is frequently overexpressed in human breast ductal carcinoma *in situ* (DCIS) specimens, which confer a high risk for the development of infiltrating ductal carcinoma. If causally involved in the genesis of human breast malignancy, cyclin D1 may represent an interesting target for chemopreventive approaches, as it sits at the junction of many growth factor and hormonal pathways. We have used the MCF-10A human breast cell line, derived from a mastectomy containing a low risk premalignant lesion, as a model system. Three cyclin D1 transfectants exhibited physiologically relevant levels of transgene overexpression, but no coordinate overexpression of other cell cycle related genes. Proliferation assays, flow cytometry, and cdk enzymatic assays of anchorage-dependent proliferation indicated only a minimal and transient effect of cyclin D1. In contrast, cyclin D1 overexpression significantly stimulated anchorage-independent colonization in soft agar or methylcellulose, accompanied by greater Gl-S progression. The cdk4 activity of the control- and cyclin D1 transfectants in colonization assays was comparable, but the cdk2 activity was higher in the latter. Injection of control- and cyclin D1 transfected MCF-10A cells in matrigel into nude mice failed to produce tumors within 1.5 years. The data suggest that cyclin D1 overexpression is an early feature of breast neoplastic progression, and can contribute to cancer development through the promotion of colonization.

Introduction

While prevention approaches to the development of hormone receptor-positive breast cancer have been developed [1], continued investigation of the molecular pathways contributing to breast malignancy will undoubtedly fuel the rational development of improved or more widely applicable preventive agents. One approach to this molecular characterization examines altered gene or protein expression levels among human breast biopsy specimens containing premalignant or ductal carcinoma *in situ* (DCIS) lesions, which have been associated with stratified risk estimates for patient development of invasive breast cancer [2–11]. We reported that 80% of DCIS specimens overexpressed cyclin D mRNA, as compared to normal ductal/lobular units in the margin of the biopsy specimen. A diagnosis of DCIS confers on the patient an 8–10 fold increased risk for the development of invasive breast cancer. Overexpression of cyclin D mRNA was infrequent in atypical ductal hyperplasia (ADH) or proliferative lesions, which confer lower risks [12]. This finding associated cyclin D overexpression with a relatively high risk for breast cancer development. Gillett et al. [13] confirmed and extended these data to the protein level, where 64% of DCIS overexpressed cyclin D1, in comparison to few lower risk ADH cases. Using a different cutpoint, where a case was

considered positive if 5% of the tumors stained, Alle et al. [14] reported that cyclin D1 expression increased from normal epithelial cells, to proliferative lesions and ultimately to DCIS. Two additional reports found cyclin D1 overexpression in DCIS, and that overexpression resulted from multiple mechanisms: gene amplification in many comedo or high grade forms, and protein overexpression in noncomedo or low grade forms [15, 16]. These observations indicate that cyclin D1 overexpression is a frequent event in DCIS, associated with a high risk estimate, and suggests the hypothesis that it functionally contributes to breast cancer development.

Cyclin D1 is one of the three members of a set of short-lived proteins which are essential activators of the cyclin-dependent kinases cdk4 and cdk2 involved in G1 cell cycle progression; the cyclin-cdk complexes can be inhibited by binding of several inhibitors ($p16^{ink4a}$, $p15^{ink4b}$, $p18^{ink4c}$, and $p19^{ink4d}$ specific for cyclin D-cdk complexes, and, $p21^{cip1}$, $p27^{kip1}$, and $p57^{kip}$ which are inhibitory to other cyclin-cdk complexes as well) (reviewed in [17, 18]). Recent reports indicate that cyclin D1 can serve additional functions, including the promotion of gene amplification in fibroblasts, participation in neuronal differentiation and apoptosis, modulation of DNA repair, direct activation of the estrogen receptor response element in breast cells, and binding of the DMP1 transcription factor [17, 19–25].

Despite years of investigation, the phenotypic role of cyclin D1 overexpression in breast cancer development remains incompletely understood. Transgenic mice overexpressing cyclin D1 tethered to a MMTV-LTR produced hyperplasias, and 8/12 mice developed mammary carcinomas with a mean latency of 551 days [26]. This hyperplastic phenotype differs from the human situation, where cyclin D overexpression was infrequent in typical or atypical hyperplasias [12, 13], and the long latency of carcinoma induction has suggested that other molecular events are required. This study underscores the need for careful analysis in human model systems. Two transfection studies using 'normal' human breast epithelial cells have been reported to date, with somewhat conflicting results – Han et al. [27] reported that cyclin D1 overexpressing HBL-100 clones were inhibited in anchorage-dependent and -independent growth and tumorigenicity. In contrast, Kamalati et al. [28] reported a growth advantage with cyclin D1 overexpression, similar to that reported in immortal fibroblasts and several other cell types. Interpretation of the data, however, was complicated by the SV40 large T antigen expression by both cell lines (which may alter the RB pathway that cyclin D1 affects), and potentially also by the variable presence of other cell cycle related proteins.

We report herein the effects of cyclin D1 transfection on the MCF-10A human mammary epithelial cell line. The MCF-10A line was derived from a mastectomy specimen containing premalignant, low risk fibrocystic changes and a focus of typical hyperplasia [29, 30]. It exhibits wild type p53, and is nontumorigenic. We report that cyclin D1 overexpression augmented anchorage-independent colonization, suggesting a contributory role to the genesis of human breast cancer.

Materials and methods

Cell culture and transfection

MCF-10A cells were grown in DMEM/F-12 (1:1) medium supplemented with 20 mM HEPES, 5% heat inactivated horse serum, 0.1 μg/ml cholera toxin, 10 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml EGF, and 0.5 μg/ml hydrocortisone. The cells were maintained in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. MCF-10A cells were infected with culture supernatants from the amphotropic packaging cell line PA317 (ATCC) transfected with pBabe retrovirus vector [31] with or without a cyclin D1 cDNA insert [32] under the control of a Mo MuLV LTR. Three cyclin D1 overexpressing clones were selected from 18 puromycin resistant colonies, based on cyclin D1 overexpression and lack of other cell cycle protein overexpression; three control transfectants (pBabe vector) were randomly selected. Where indicated, pools of control- or cyclin D1 transfectants were used, and were made by combining equal numbers of cells from the three clonal transfectants.

The KE and GH mutant forms of cyclin D1 [33] were subcloned into the pBabe vector. Virus supernatants from PA317 cells, which had been transfected with pBabe, pBabe-cyclin D1, pBabe-cyclin D1KE, and pBabecyclin D1GH were used to infect MCF-10A cells, as described above.

Cell proliferation assay

To assay anchorage-dependent growth, 200 MCF-10A cells in 100 μl medium were plated into each well of

96-well plates, which were coated in some experiments with fibronectin or matrigel. Cell proliferation was determined using the Cell Titer 96 TM kit according to the manufacturer's instructions (Promega) [34].

Anchorage-independent proliferation assay

Cell anchorage-independent growth was determined by soft agar [35] and methylcellulose culture [36]. For the soft agar assay, $0.5 - 1 \times 10^4$ cells/ml of MCF-10A control and cyclin D1 transfectants were grown in 0.5 ml of 0.3% Difco agar (Difco) in MCF-10A growth medium. The cell suspensions were layered over 0.5 ml of 0.7% bottom agar in 24-well plate. After two weeks of culture the colonies (>50 cells) in soft agar were counted using a grid on the bottom of the dish and a microscope. Each point represents the mean of three cultures and each experiment shown is representative of at least three conducted.

Cell cycle analysis

For monolayer culture, 2×10^5 cells per 60 mm culture dish of MCF-10A control and cyclin D1 transfectants were trypsinized and flow cytometric analysis was conducted on day 3 using propidium iodide staining on a Becton Dickinson FACScan flow cytometer [37]. For methylcellulose experiments, 1×10^6 cells from exponential cultures of MCF-10A control or cyclin D1 transfectants were grown in 8 ml of 1% methylcellulose (Sigma) in 15-ml conical tubes and harvested after 24 h of culture for flow cytometry.

Immunoprecipitation and western blot analysis

5×10^5 cells per 100 mm culture dish of MCF-10A control- or cyclin D1 transfectants were cultured for 2.5 days before they were lysed in buffer 1 (PBS with 1% NP-40, 1 mM PMSF, 5 μg/ml aprotinin, 5 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM sodium vanadate, and 5 mM EDTA). Cellular lysates equivalent to 60 or 100 μg protein were resolved on an 8–16% SDS–PAGE and were transferred to a nitrocellulose membrane. Western blots were performed using 1 μg/ml antibodies incubated overnight at 4°C and detected by chemiluminescence (DuPont NEN or Pierce). The antibodies to cyclin D1, cyclin D3, p21, p27, p16 (Lab. Vision), and cyclin E, Cdk2, Cdk4, Cdk6, p53 (Santa Cruz) were used. For immunoprecipitations, 500 μg lysates were precleared with protein A-sepharose (Pharmacia LKB) for 1 h, and the supernatants were incubated overnight at 4°C with 5 μg cyclin D1 polyclonal antibody (Upstate). The immune complexes were precipitated by protein A-sepharose and resolved on an 8–16% SDS–PAGE which was transferred to nitrocellulose. The cell cycle related proteins in cyclin D1 complex were detected by western blot.

Cyclin D1 kinase (cdk 4) assay

Exponential cultures of MCF-10A control or cyclin D1 transfectants were lysed in lysis buffer 2 (50 mM HEPES, pH7.5, containing 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween-20, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 5 μg/ml pepstatin, 1 mM NaF, 10 μg/ml aprotinin, 10 mM β-glycerophosphate, and 0.1 mM sodium vanadate). Five hundred microgram lysates were precleared with protein A-sepharose before the supernatants were incubated with 5 μg cyclin D1 antibody (Upstate) for 3 h at 4°C. The immune complexes were precipitated by protein A-sepharose and cyclin D1 kinase activity was determined using 0.45 μg GST-Rb (Santa Cruz) and 10 μCi [γ-^{32}P]ATP (ICN) as described previously [37].

Cdk 2 kinase assay

Exponential cultures of MCF-10A control- or cyclin D1 transfectants were lysed in lysis buffer 3 (50 mM Tris–HCl, pH 7.5, containing 250 mM NaCl, 0.1% TritonX-100) with 0.1 mM PMSF, 5 μg/ml pepstatin, 1 mM NaF, 10 μg/ml aprotinin, 10 mM B-glycerophosphate, 0.1 mM sodium vanadate, and 1 mM EDTA. Five hundred micrograms of lysate was incubated with 2 μg anti-cdk2 (Santa Cruz) which had been precoated to protein A-sepharose for 3 h at 4°C. Cdk2 activity was determined using 5 μg histone H1 and 10 μCi [γ-^{32}P] ATP (ICN) as described previously [38].

Tumorigenicity assay using nude mice

Each of MCF-10A control and cyclin D1 transfectants was injected subcutaneously into ten 4–6 week old female NIH nu/nu mice. 1×10^7 cells suspended in 0.1 ml of matrigel were injected into the right and left dorsal flanks of the mouse [39]. After subcutaneous injection, animals were checked for palpable lesions at weekly intervals. All *in vivo* procedures were per-

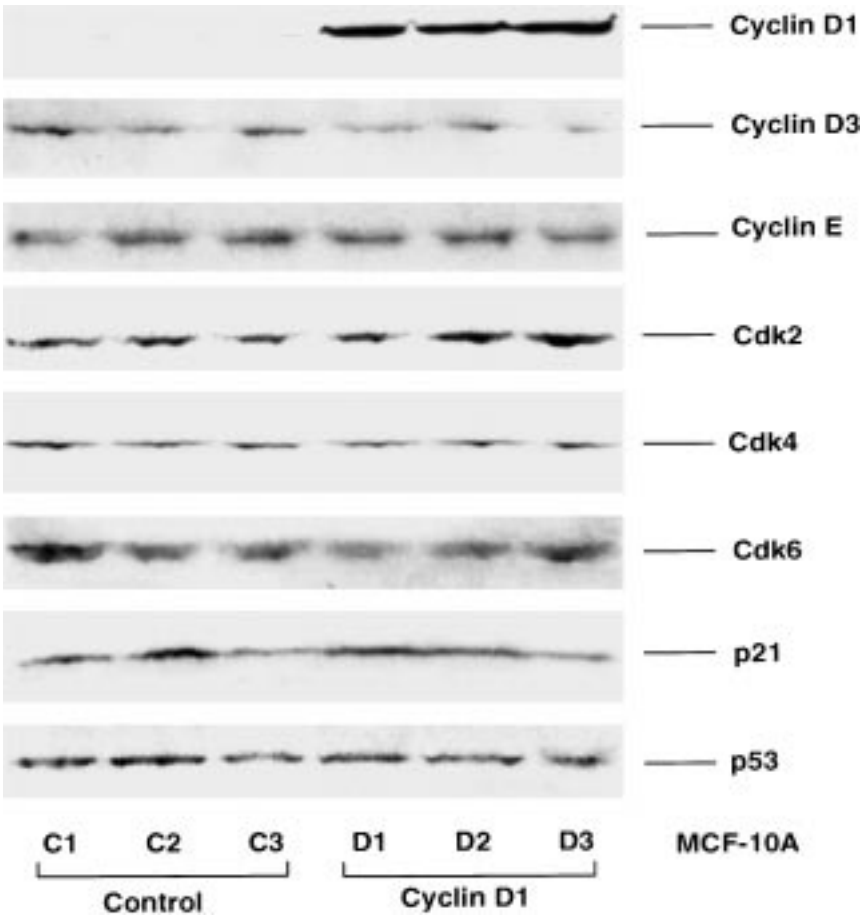

Figure 1. Cyclin D1, but not other cell cycle proteins, is overexpressed in anchorage-dependent cultures of transfected human MCF-10A breast clones. Western blots of total cell lysates from three independent cyclin D1 transfectants (D1, D2, and D3) and three independent control transfectants (Cl, C2, C3) are shown for multiple proteins.

formed in compliance with approved NIH animal use proposal LP-008.

Results

Characterization of cyclin D1-transfected MCF-10A breast cells

MCF-10A cells were transfected with a pBabe retroviral vector containing a cyclin D1 cDNA or empty insert. Figure 1 shows the cyclin D1 protein levels among three independent control clones (Cl, C2, C3) and three cyclin D1 transfectants (D1, D2, D3). Five–ten fold overexpression was noted in the cyclin D1 transfectants. Because studies have reported the overexpression of other cell cycle related genes coincident with cyclin D1 transfection (27, 40), clones were analyzed for cyclin, cdk, and cdk-inhibitor protein levels by western blot analysis. We did not find frequent overexpression of other cell-cycle proteins in the majority of the MCF-10A clonal transfectants. As shown in Figure 1, cyclins D3 and E, cdks 2, 4 and 6, p21, and p53 were expressed at comparable levels in the two sets of transfected clones. p27 was difficult to visualize in 100 μg of cell lysate but appeared equally expressed (data not shown); other proteins which were undetectable in 100 μg of cell lysate included cyclin D2 and p16.

Effect of cyclin D1 transfection on anchorage-dependent growth

In the experiments shown in Figure 2, MCF-10A clones were cultured under various conditions in microtiter wells, and anchorage-dependent growth quantitated using a MTT assay. At normal serum concen-

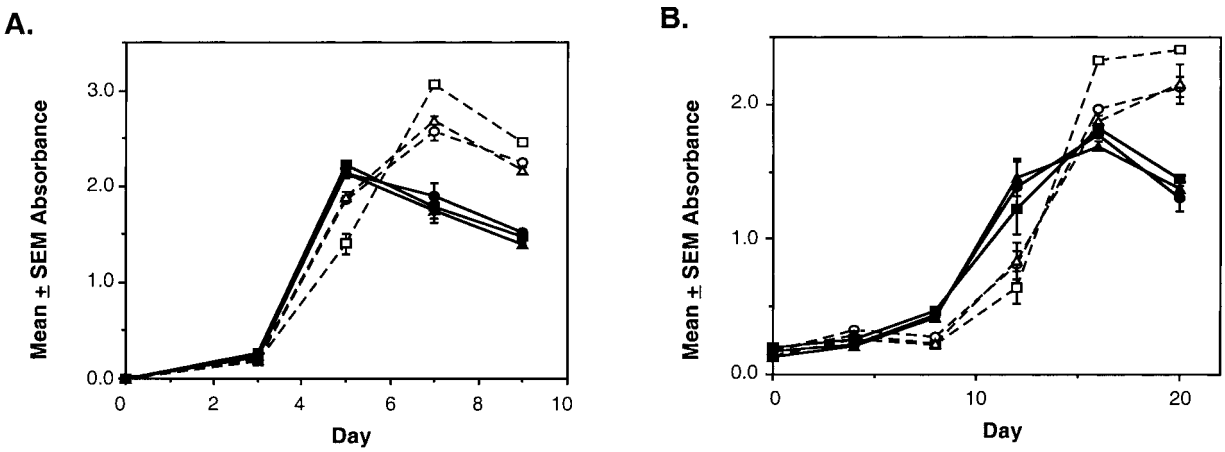

Figure 2. Cyclin D1 overexpression induces only a transient increase in anchorage-dependent growth in MCF-10A breast cells. The anchorage-dependent growth of three independent control transfectants (Cl, C2, and C3, dashed lines) and three independent cyclin D1 transfectants (D1, D2, and D3, solid lines) was determined on multiple days of culture using a spectrophotometric assay. The mean ± sem absorbance of triplicate cultures at each timepoint is graphed under two culture conditions: (A) Normal (10%) serum; (B) Low (0.1%) serum. Cyclin D1 transfectants significantly varied from the control transfectants ($p < 0.01$, student's t-test): Panel A, days 5,7,9; Panel B: days 12, 20.

trations (Figure 2A), each of the three independent cyclin D1 transfectants (solid lines) exhibited a slight (30%) increase in cell density through the exponential phase of growth (days 3–5 of culture), as compared to control transfectants (dashed lines). This increase was not maintained, as the cyclin D1 transfectants subsequently exhibited a 30% decrease in cell number as compared to the control transfectants on days 7–9 of culture. Similar trends were observed if the cells were cultured on fibronectin or matrigel (data not shown). Growth in low serum media is often considered an assay of growth factor independence. The cyclin D1 transfectants exhibited similar anchorage-dependent growth trends in 0.5% serum, but with a longer time course (20 days) and a maximum of 2-fold growth stimulation over controls (Figure 2B).

The relatively minor growth advantage of the cyclin D1 transfectants was mirrored in their cell cycle distribution (Table 1). Flow cytometry of each clone on day 1 of culture indicated that 33–35% of the cyclin D1 transfectants were in S-phase, as compared to 24–30% of control transfectants. The increased S-phase expression of the cyclin D1 transfectants was at the expense of G0/G1 participation. G2/M levels remained comparable in all of the transfectants.

Increased cyclin D1 was observed in the D1–D3 clones upon immunoprecipitation (Figure 3), confirming the western blot data. The increased amount of cyclin D1 in these clones was associated with a 2-fold increase in cdk4 binding, and a 4-fold increase in the amount of p21 bound. Low but comparable amounts of p27 were found in the cyclin D1 and control transfectant complexes (data not shown). The enzymatic activities of the cyclin D1-cdk complexes from each transfectant were assayed by immunoprecipitation and kinase assays (Figure 3B). When anti-cyclin D1 immunoprecipitated complexes were incubated with ^{32}P-γATP and RB in an assay of cdk4 function, a minor (25% by densitometry) but repeatable increase in RB kinase activity was observed in the cyclin D1 transfectants. The RB phosphorylation status of the control- and cyclin D1 transfectants was comparable on western blots (data not shown). Cyclin D1 has been reported to indirectly affect Cdk2 activity, via its binding of common cdk inhibitors [41]. When lysates were immunoprecipitated with anti-Cdk2 and tested in a histone kinase assay, comparable activity was demonstrated in all lysates (Figure 3B) suggesting that this

Table 1. Cell cycle distribution of control- and cyclin D1 transfected MCF-10A breast cells under anchorage-dependent culture conditions

Clone	G0 + G1 (%)	S (%)	G2 + M (%)
C1	50.5	30.4	19.1
C2	52.0	24.5	23.5
C3	52.1	28.4	19.5
D1	43.9	33.6	22.5
D2	44.9	34.9	20.2
D3	47.1	34.2	18.8

Cells were harvested from semiconfluent cultures and the cell cycle distribution analyzed by flow cytometry. Data are from a single culture of each transfectant, and representative of three independent experiments conducted.

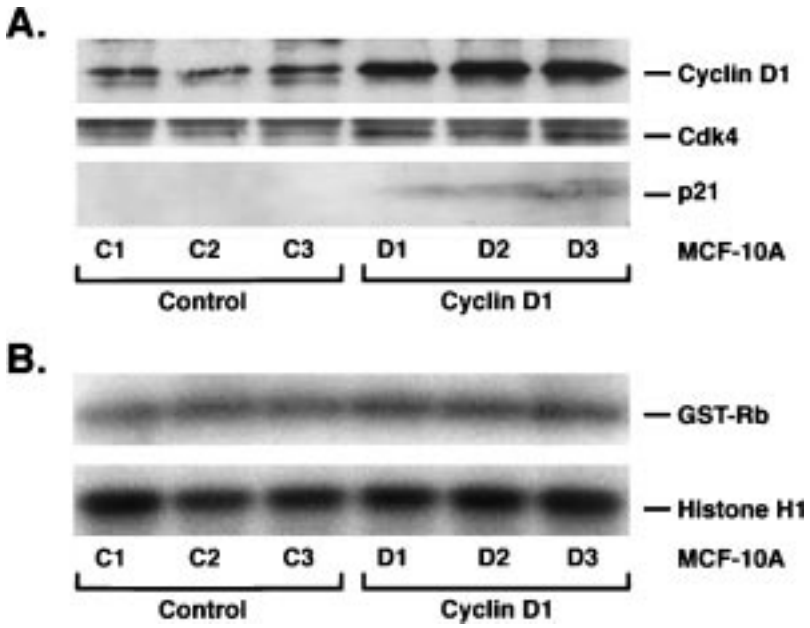

Figure 3. Cyclin D1 complex formation and cdk enzymatic activities under anchorage-dependent conditions. A. Cell lysates from three control transfectants (Cl, C2, and C3) and three cyclin D1 transfectants (D1, D2, and D3) were immunoprecipitated with anticyclin D1, and proteins in the complex resolved by western blot analysis. B. Anti-cyclin D1 or anti-cdk2 immunoprecipitated cell lysates were assayed for kinase activity using GST-RB and Histone as substrates, respectively.

titration of cdk inhibitors had not occurred. Taken together, the data indicate that overexpression of cyclin D1 in the MCF-10A breast cell line resulted in a minor and ephemeral increase in anchorage-dependent growth, which was correlated with a minor increase in cdk4 activity.

Effect of cyclin D1 transfection on anchorage-independent growth

In contrast to anchorage-dependent assays, a significant increase in anchorage-independent colonization was observed in each of the cyclin D1 transfectants. This increase was observed using either 0.3% agar (Figure 4A) or 1.0% methylcellulose (Figure 4B) in the cultures, the latter of which permitted harvest of the cells for subsequent assays. A photograph showing representative colonies formed by the cyclin D1 transfectants is included as Figure 4C; the three-dimensional nature of the soft-agar culture necessarily makes some colonies poorly focused. The increased colonization potential of the cyclin D1 transfectants was maintained through the limit of our culture conditions, approximately five weeks.

Flow cytometry of control- and cyclin D1 transfectants harvested from methylcellulose cultures is summarized in Table 2. Overall levels of S-phase participation were lower than under anchorage-dependent conditions (Table 1), with a greater percentage of cells in G0/G1. However, the cyclin D1 transfectants contained 18–21% of cells in S-phase, as compared to 9–10% of control transfectants. This represents a larger difference than under anchorage-dependent conditions.

In order to investigate the mechanism of anchorage-independent colonization further, we determined the relative expression levels of cell-cycle proteins in total cell lysates from methylcellulose cultures. While cyclin D1 was overexpressed in the D1–3 transfectants, comparable levels of cyclins D3 and E, cdks 2, 4 and 6, p16, p21, p53, and p27 were observed in the cyclin D1 and control-transfectants (data not shown). Immunoprecipitation of cyclin D1 from anchorage-independent cultures and western blot analysis of complexes is shown in Figure 5A. The immunoprecipitations resemble those conducted under anchorage-dependent conditions, exhibiting a relatively minor increase in cdk4 binding, and a substantial increase in p21 binding. Enzymatic activities of the lysates under anchorage-independent conditions are shown in Figure 5B. The cdk4 activity of the transfectants was comparable. In data not shown, the Rb protein level and phosphorylation status were also comparable. In a significant diversion from data under

anchorage-dependent conditions, the cdk2 activity of the cyclin D1 transfectants was present, and virtually undetectable in the control transfectants. Thus, the increased anchorage-independent colonization of cyclin D1 overexpressing human MCF-10A clones was associated with a relative increase in cdk2 activity. While western blot data established that the control- and cyclin D1 transfectants expressed comparable amounts of p21, we asked if overall levels of p21 varied between culture conditions. The western blot shown in Figure 5C indicates that, while p21 levels were comparable between the control- and cyclin D1 pooled transfectants, p21 was decreased by anchorage-independent culture conditions.

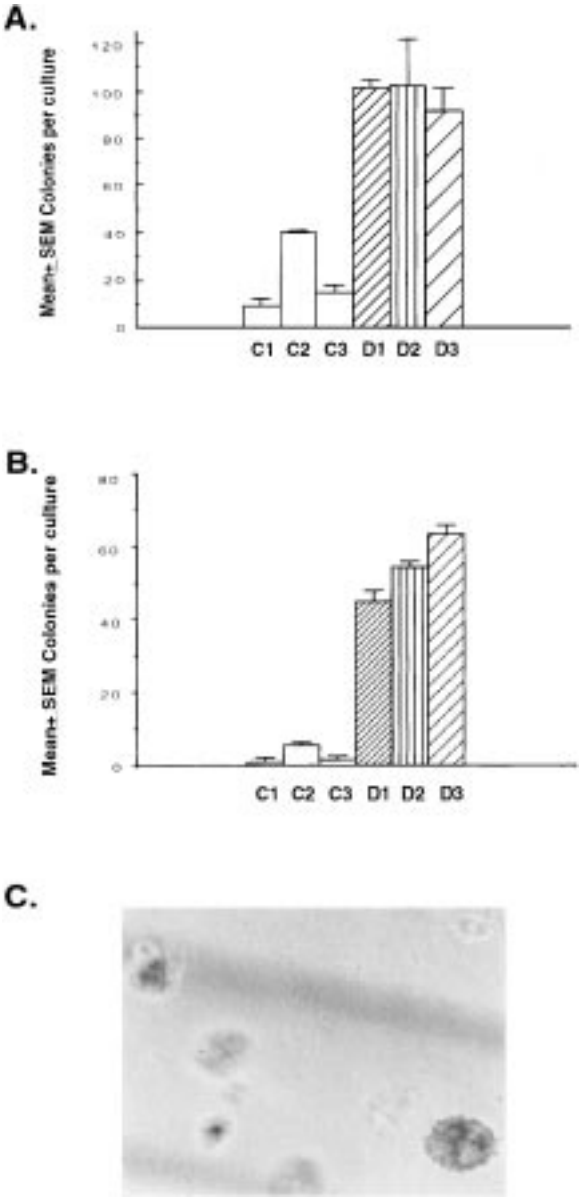

Figure 4. Cyclin D1 overexpression by MCF-10A breast cells increases anchorage-independent colonization. The colonization of control transfectants (open bars) and cyclin D1 transfectants (filled bars) in anchorage-independent assays using 0.3% soft agar (Panel A) or 1% methylcellulose (Panel B) was determined after 2 weeks of culture. Each point represents the mean ± sem of triplicate cultures. The colonization of the control- and cyclin D1 transfectants was significantly different, $P < 0.01$, student's *t*-test. Panel C shows a representative photomicrograph of soft agar colonies produced by the cyclin D1 transfectant.

Table 2. Cell cycle distribution of control- and cyclin D1 transfected MCF-10A breast cells under anchorage-independent culture conditions

Clone	G0 + G1 (%)	S (%)	G2 + M (%)
C1	76.4	8.9	14.7
C2	74.6	9.6	15.8
C3	76.0	9.8	14.2
D1	65.3	17.9	16.8
D2	63.3	21.3	15.4
D3	62.8	20.4	16.8

Cells were harvested after 24 h culture in methylcellulose, and the cell cycle distribution analyzed by flow cytometry. Data were obtained from a single culture of each transfectant, and are representative of three independent experiments conducted.

Site directed mutagenesis of cyclin D1

MCF-10A cells were transfected side-by-side with a control vector, wild type cyclin D1, and two cyclin D1 mutant constructs. The KE mutation alters the 'cyclin box' of cyclin D1 such that interaction with cdk4 is inhibited [33]; the GH mutation abrogates cyclin D1 binding to the RB protein [32]. Three clones expressing each mutant transfectant were compared to control and wild type transfectants (Figure 6A). Expression of the KE mutant was high among 14 clones analyzed, and three representative independent clones were selected; expression levels of the GH mutant were lower overall. Three wild type cyclin D1 clones (D4–D6) were selected which reflected the range of overall protein expression, and three control clones were randomly selected (C4–C6).

The colonization potential of each clone is graphed in Figure 6B. Three wild type cyclin D1 transfectants produced significantly more colonies than control transfectants. Colonization by KE overexpressing clones was comparable to that of the control transfectants, implicating the cdk binding and/or activity

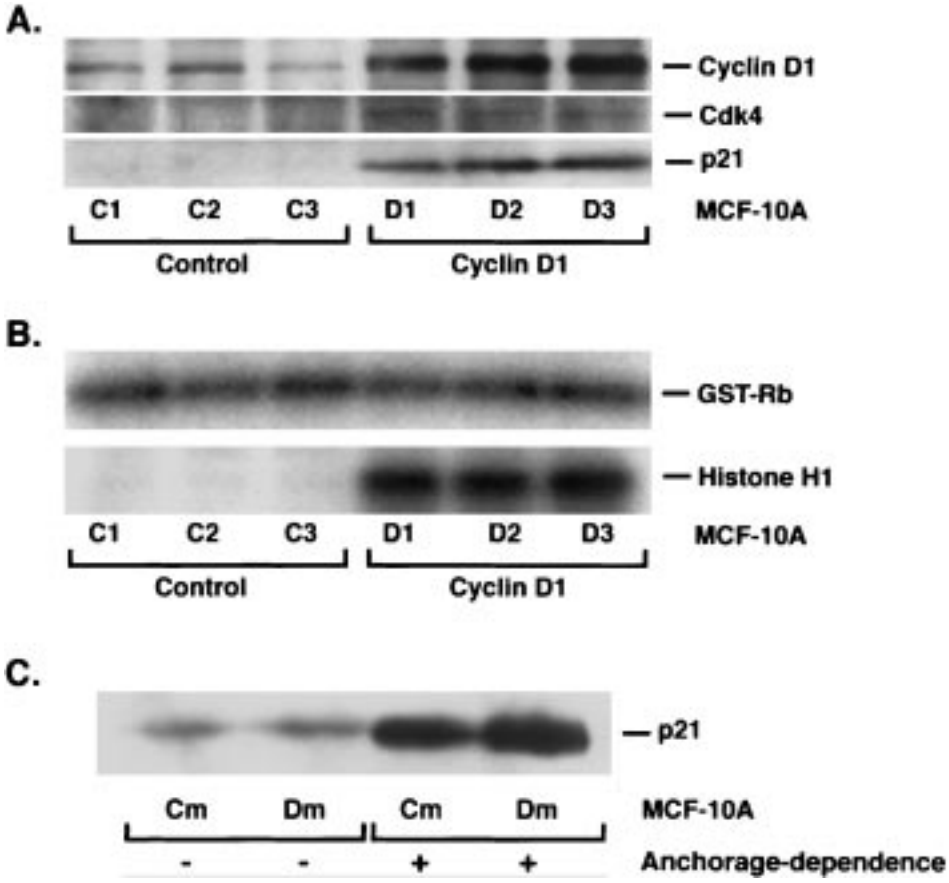

Figure 5. Cyclin D1 complex formation and cdk enzymatic activities under anchorage-independent conditions. A. Cell lysates from three control transfectants (C1, C2, and C3) and three cyclin D1 transfectants (D1, D2, and D3) were immunoprecipitated with anticyclin D1, and proteins in the complex resolved by western blot analysis. B. Anti-cyclin D1 or anti-cdk2 immunoprecipitated cell lysates were assayed for kinase activity using GST-RB and histone as substrates, respectively. C. Pools of the three control transfectants (Cm) and three cyclin D1 transfectants (Dm) were grown under anchorage-dependent and -independent conditions, harvested, and western blot analysis of p21 conducted.

of cyclin D1 in its colonization potential in this model system. However, destruction of the RB binding motif in cyclin D1 did not inhibit colonization. Thus, the cyclin D1-cdk interactive and not the RB associative properties of cyclin D1 were required for its potentiation of colonization.

In vivo *growth of the cyclin D1 transfectants*

Injection of a bolus ($\geq 10^7$ cells) of the parent MCF-10A line into mice, either in the presence or absence of matrigel, resulted in the presence of small palpable nodules which disappeared after several weeks. Derivatives of this line containing mutated *ras* have occasionally produced premalignant lesions and tumors when injected sc in matrigel [29], so this procedure was adopted to test the tumorigenicity of the cyclin D1 and control transfectants. Figure 7 shows the results of one of two experiments conducted, in which 10^7 cells were injected sc within matrigel in each flank of the mice. The initial lesion size at injection was comparable between the control- and cyclin D1 transfected clones. The lesions from all of the transfectants disappeared by week 8. At one year postinjection no lesions appeared palpable, and histological examination of H&E stained sections from the injection site contained only normal mammary fat-pad structures (personal communication, Drs. Maria Merino and Gil Smith, NCI). Thus, despite increased colonization potential, the cyclin D1 transfectants remained nontumorigenic.

Discussion

In order to determine the contribution of cyclin D1 overexpression to the genesis of human breast can-

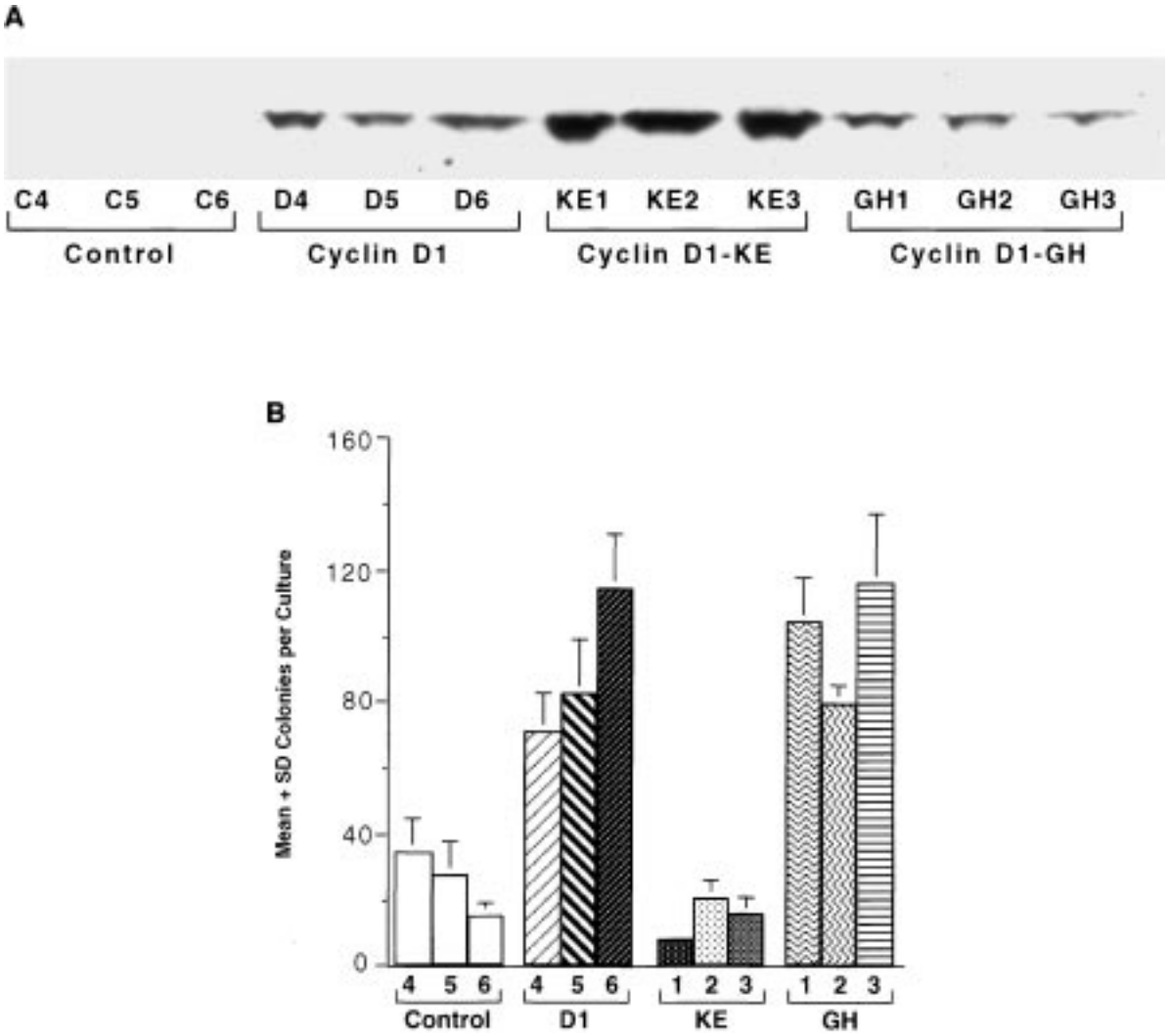

Figure 6. Site directed mutagenesis of cyclin D1 reveals that its cdk association, but not RB interaction, are required for increased colonization. MCF-10A cells were transfected, and overexpressing independent clones identified for a control vector (C4, C5, and C6), wild type cyclin D1 (D4, D5, and D6), the KE mutant of cyclin D1 involved in the cyclin box (KE1, KE2, and KE3) (significantly different from cyclin D1 transfectants, $p < 0.05$, student's *t*-test), and the GH mutant of cyclin D1 involved in RB binding (GH1, GH2, and GH3). Panel A: Total cyclin D1 protein levels determined by western blot. Panel B: Anchorage-independent colonization in soft agar, with each bar representing the mean $\pm$ sem of triplicate cultures.

cer, model systems which faithfully reflect the disease process must be developed. Several features of the model system described herein have been optimized. First, cyclin D1 overexpression was obtained in the range of 5–10 fold. When grains of *in situ* hybridization were counted in our cohort study, the minimal difference denoting overexpression was 2–3 fold, and many lesions were double this, in the 4–9 fold range [12]. Thus, the degree of overexpression in this transfection study was consistent with values observed in nature. Second, the transfected MCF-10A clones did not vary in overall levels of a number of other cell cycle related genes, including other cyclins, cdks, cdk inhibitors, and p53. Secondary or random changes in gene expression have hampered the interpretation of other cyclin D1 transfection studies [27, 40]. Third, the recipient cell line, MCF-10A, was derived from a mastectomy specimen containing low risk premalignant breast disease, and approximates the premalignant phenotype. MCF-10A cells exhibit advanced features such as immortalization, but normal phenotypes in p53, tumorigenicity, etc. To our knowledge,

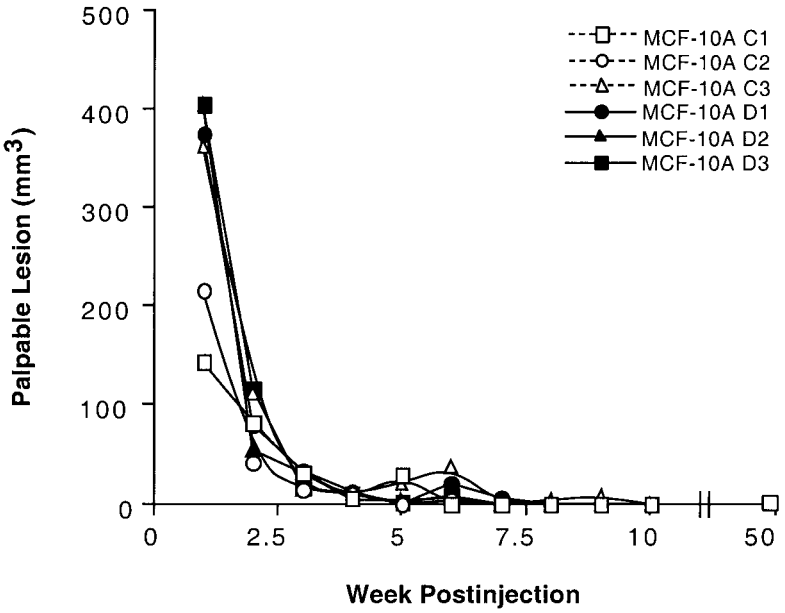

Figure 7. Cyclin D1 overexpression by human MCF-10A breast cells is insufficient to produce tumorigenicity *in vivo*. Three control transfectants (C1, C2, and C3, open symbols) and three cyclin D1 transfectants (D1, D2, and D3, closed symbols) were injected sc in matrigel into each flank of nu/nu mice, and lesion size estimated as $l \times w \times h$, determined weekly by calipers, for one year.

no cell lines exist from a DCIS lesion. Fourth, the use of methylcellulose cultures for anchorage-independent assays has enabled some of the first molecular characterization of this complex phenomenon. The model can be extended in future studies in several directions. For instance, the additive or synergistic role of genes whose altered expression is prevalent in comedo DCIS would be of interest. Candidates include erb-B-2/Her-2/neu, mutated p53, angiogenesis stimulators and inhibitors, etc. Also, studies for other functions of cyclin D1 not directly related to G1 progression, are of interest to determine if they also affect the breast cancer phenotype.

We present a characterization of the growth effects of cyclin D1 transfection *in vitro* and *in vivo*. The most salient finding is that overexpression of cyclin D1 significantly increased anchorage-independent colonization of the MCF-10A cells. This can be postulated to promote colonization of 'normal' epithelial cells in the breast architecture, which may contribute to their neoplastic progression. In this model system overexpression of cyclin D1 failed to promote full tumorigenicity within a period of 1.5 years. This would be consistent with transgenic mouse data if the long latency and focal nature of the mammary carcinomas were due to a requirement for additional molecular events [26, 42]. Thus, if our model system is relevant to human breast cancer, it is likely that cyclin D1 overexpression is contributory but not sufficient.

The complexity of cyclin D1 action is suggested by its stimulation of anchorage-independent, but not dependent, growth in our model system. Similar trends have been reported for cyclin D1 overexpression in other cell types, including rodent fibroblasts and human gliomas [43], suggesting the generality of this phenotype. Other cell cycle genes have also exerted varying effects on anchorage-independent and -dependent growth in transfection studies, but the mechanism of differential regulation was unknown [44, 45]. Overall levels of many cell-cycle proteins as well as those proteins binding cyclin D1 were examined from both anchorage-dependent and methylcellulose cultures, and appeared comparable between the control- and cyclin D1 transfectants. Our data from anchorage-dependent conditions indicated that cdk4 activity was not significantly increased by cyclin D1 overexpression, perhaps because of the additional p21 binding to the complex. Based on the site-directed mutation data, the binding of cyclin D1 to cdks was necessary for colonization, however. The formation of a cyclin D1-cdk complex is now known to require additional proteins, including p21 and/or p27 [46–48]. Given the observation that the enzymatic activity of this complex was not dramatically changed, the

data suggest that the binding activity of the complex may be important. Another potential contributor to the anchorage-dependent data is a block in S-phase, which could involve additional unstudied mechanisms.

The most profound difference between the anchorage-dependent and -independent cultures lies in the dramatic difference in cdk2 activity between the cyclin D1 and control transfectants under the latter culture conditions. The loss of normal, but not transformed, cell growth when changed to anchorage-independence has been previously correlated with reduced cdk2, kinase activity [49]. One explanation for the observed data is that two events, (1) increased cyclin D1 which bound p21 and cdk4, and (2) an overall decrease in p21 levels in anchorage-independent conditions, served to titrate-out free p21, resulting in enhanced cdk2 activity. Other proteins unstudied herein may also contribute to the observed colonization. A similar sequestration of p21, with concomitant activation of cdk2, was recently proposed to mediate estrogen induced growth of breast cells [50, 51]. p21 has also been reported to participate in other pathways not directly involved in Gl progression, including DNA repair, centrosome duplication, and mRNA splicing [52–54], and these pathways may be participatory. Immunohistochemical studies have detected heterogeneous expression and potential mutations of p21 in human breast DCIS specimens [55, 56], suggesting the potential relevance of this pathway to the human disease. Several reports have found opposite trends, whereby overexpression of cyclin D1 induced p21 expression [43], or where anchorage-independence resulted in increased p21 expression [57, 58]. Many of these findings were mediated by p53, and it may be that p53-dependent and -independent pathways exist.

If confirmed in additional human model systems, cyclin D1 may represent an interesting target for pilot breast cancer chemoprevention studies. Our data suggesting a facilitation of Gl-S progression in colonization represents a point at which many growth factor and hormonal signaling pathways converge, and thus may be more widely applicable, including the estrogen-receptor negative subpopulation for which we have no effective chemoprevention regimens yet. Indeed, our laboratory and others have reported that retinoids, which are being considered in a chemopreventative setting, decrease breast tumor cell line cyclin D1 expression *in vitro* [37, 59, 60].

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