In normal cells, cyclin D1 is induced by growth factors and promotes progression through the G1 phase of the cell cycle. Cyclin D1 is also an oncogene that is thought to act primarily by bypassing the requirement for mitogens during the G1 phase. Studies of clinical tumors have found that cyclin D1 overexpression is associated with chromosomal abnormalities, although a causal effect has not been established in experimental systems. In this study, we found that transient expression of cyclin D1 in normal hepatocytes in vivo triggered dysplastic mitoses, accumulation of supernumerary centrosomes, abnormalities of the mitotic spindle, and marked chromosome changes within several days. This was associated with up-regulation of checkpoint genes p53 and p21 as well as hepatocyte apoptosis in the liver. Transient transfection of cyclin D1 also induced centrosome and mitotic spindle abnormalities in breast epithelial cells, suggesting that this may be a generalized effect. These results indicate that cyclin D1 can induce deregulation of the mitotic apparatus and aneuploidy, effects that could contribute to the role of this oncogene in malignancy.

A critical component of normal cell division is the accurate distribution of chromosomes and other cellular components during mitosis. Abnormal cell division and chromosome content are hallmarks of cancer and are associated with a poor prognosis in a number of tumors (1, 2). The mechanisms by which cells acquire chromosome changes have not been fully identified, but alterations of centrosomes and the mitotic spindle apparatus appear to play an important role (3–6). Most normal cells contain one centrosome, which serves as the major microtubule organizing center and participates in processes such as cell polarity, migration, and intracellular transport (3–7). In normal cell division, centrosomes undergo one round of duplication in a manner analogous to the replication of chromosomal DNA during S phase. During mitosis, centrosomes direct the formation of bipolar mitotic spindles that ensure equal segregation of chromosomes between daughter cells.

Increased numbers of centrosomes are frequently observed in malignant cells (3–7). This is thought to result in distortion of the mitotic apparatus and abnormal sorting of chromosomes during cell division. The resulting changes in chromosome complement can lead to loss of tumor suppressor genes or gain of oncogene function that further promote the malignant phenotype. In addition to changes in centrosome number, alterations in the centrioles and pericentriolar material that make up the centrosome are frequently seen in cancers. Although recent studies have provided substantial insight into the proteins that make up the centrosome, the identity and function of centrosome components, and their potential derangement in cancer, remain to be fully characterized.

Recent studies have identified proteins that govern the centrosome duplication cycle (reviewed in Refs. 5 and 7–11). The activity of cyclin-dependent kinase (cdk)3 2 is thought to be required for centrosome duplication in tissue culture and cell-free systems. Relevant substrates of cdk2 include the Mps1 kinase, nucleophosmin/B23, and CP110, which regulate centrosome duplication. The cdk inhibitor p21, which is a downstream target of p53, is thought to inhibit the centrosome cycle through inactivation of cdk2 activity. Cells lacking p53 function may also be deficient in other cellular checkpoint mechanisms that affect centrosome duplication. In addition to p53 and p21, mutations of tumor suppressor genes, Brca1, Brca2, and Gadd45, and adenomatous polyposis coli are associated with centrosome abnormalities. Conversely, overexpression of several oncogenes that regulate the mitotic spindle apparatus, such as Ran and Aurora-A, can disrupt normal centrosome function.

Cyclin D1 is a G1 phase regulatory protein that promotes physiologic cell proliferation downstream of mitogens and other extracellular stimuli (12–14). In addition, cyclin D1 is a putative oncogene that is overexpressed in many human malignancies. Constitutive expression of cyclin D1 is likely to contribute to malignant transformation by reducing the dependence on extracellular signals that normally control proliferation, i.e. it diminishes the requirement for mitogens in the transition through the G1 restriction point. Studies of clinical tumors have found that cyclin D1 overexpression is associated with chromosomal abnormalities (15–18), although data indicating
that cyclin D1 can cause mitotic or chromosome abnormalities are lacking. Here we document that transient cyclin D1 expression induced centrosome amplification, deregulation of the mitotic spindle, and overt chromosome abnormalities within a matter of days. These results suggest that cyclin D1 may promote malignancy by causing genomic instability.

**EXPERIMENTAL PROCEDURES**

**Animals**—All animal studies were completed following IACUC-approved procedures and National Institutes of Health guidelines. Eight-week-old male BALB/c (Harlan Sprague-Dawley), or p21−/− or p21+/+ (The Jackson Laboratories) mice were injected with 5 × 10⁶ plaque-forming units via tail vein injection of E1-deleted recombinant adenoviruses encoding cyclin D1, cyclin E, or β-galactosidase (control) as described previously (19–22), followed by liver harvest and processing. The construction of the cyclin D1 (ADV-D1) and cyclin E (ADV-E) adenoviruses is described in prior articles (19, 23, 24); the control adenovirus is equivalent except for the encoded transgene.

**Cell Culture and Immunohistochemistry**—Mouse hepatocytes were isolated using Liver Perfusion Media and Liver Digest Media (Invitrogen). Cells were purified through a Percoll gradient and cultured for the indicated times on collagen-treated glass coverslips in Williams E media in the presence of epidermal growth factor (10 ng/ml) and insulin (20 milligrams/ml) (23). Media were changed daily; colcemid (25 ng/ml) was added 3 h prior to fixation. Immunohistochemistry was completed on coverslips with attached cells after being fixed in −20 °C methanol and then rehydrated in phosphate-buffered saline containing 0.05% Tween 20. A mixture of primary antibodies, mouse monoclonal anti-α-tubulin (Sigma), and rabbit polyclonal anti-Cep135 or anti-Cep135 and anti-centrin (gift of Dr. J. Salisbury) were incubated as described previously (25, 26). hMEC cells were incubated with monoclonal anti-α-tubulin and rabbit polyclonal anti-γ-tubulin (Sigma) (6, 27). Appropriate secondary antibodies were used to visualize staining. Microscopic identification was completed by using a Nikon eclipse microscope with epi-fluorescence optics or a Zeiss LSM 510 confocal microscope. In Fig. 2E, slides were stained with DAPI, and the percentage of cells in metaphase was counted in three different specimens for each condition.

**Apoptosis and Flow Cytometry**—TUNEL staining on formalin-fixed liver tissue sections was performed by using the ApoTag (Intergen) kit following the manufacturer’s instructions, as described previously (19). Flow cytometry was completed using methods described previously (19, 21).

**Quantification of p21 and p53 mRNA by Real Time PCR (RT-PCR)**—Total RNA from each liver was isolated as described previously (28). RNA samples were subjected to agarose gel electrophoresis and visualized using ethidium bromide to ensure that the RNA was not grossly degraded. Samples of RNA (5 μg) were treated with DNase I (DNase-free; Ambion) according to the manufacturer’s instructions. Oligo(dT)-primed cDNA was generated from 4 μg of each RNA with Taqman reverse transcriptase reagent kit (Applied Biosystems). Mouse p21 DNA sequences for upper (5′-cgggtggaactttgacttcgt-3′) and lower (5′-caggcagaggaatctg-3′) primers, p53 upper (5′-agagaagcgctaca-gaga-3′) and lower (5′-ctgtagatggcccattcctt-3′) primers, and β-actin sequences for upper (5′-aactatagccaatgctga-3′) and lower (5′-acctctctggtgaatagca-3′) primers were selected by using the Primer sequence were synthesized at the University of Minnesota microchemical facility and purified by high pressure liquid chromatography. RT-PCR was completed using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Applied Sciences). Samples were denatured for 10 min at 95 °C and then 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 68 °C for 20 s. Optimization of MgCl₂ and primer concentrations was completed as recommended by the manufacturer (2.4 mM MgCl₂ for p21 and p53 and 3 mM for β-actin). Primer concentrations were found to be optimal at 0.2 μM for p21 and p53 and 0.1 μM for β-actin. For each mRNA, quantification was completed by comparison (linear interpolation) of the cycles to saturation in each sample. p21 and p53 mRNA were normalized to β-actin mRNA expression (which did not change under any of the conditions, data not shown), and the relative amounts were determined as recommended by the manufacturer.

**Transfection of Human Mammary Epithelial Cells (hMEC)—hMEC cells were transfected with the cyclin D1 or control adenoviruses (25 plaque-forming units per cell), seeded on glass coverslips, and harvested at 48 h as described previously (27).**

![Fig. 1. Cyclin D1 induces abnormal hepatocyte mitoses in vivo.](http://www.jbc.org/)

**RESULTS**

**Persistent Expression of Cyclin D1 Promotes Abnormal Hepatocyte Mitoses in Vivo**—Previous studies (21–23, 29–31) have suggested that cyclin D1 plays an important role in regulating hepatocyte proliferation in response to extracellular stimuli. In normal liver, hepatocytes rarely replicate, but these cells rapidly enter the cell cycle in response to injuries that diminish functional hepatic mass (31, 32). We have found that transient transfection of hepatocytes in vivo with a recombinant adenovirus expressing human cyclin D1 (ADV-D1) is sufficient to trigger hepatocyte replication and liver growth under conditions where these cells are normally quiescent (20–22). Intravenously injected replication-defective (E1-deleted) adenoviruses such as these primarily target the liver and can transfect >95% of hepatocytes; this system has been used extensively to study the effect of transient single-gene expression in these cells (24, 33–35). Two days after cyclin D1 transfection, hepatocyte mitoses appeared normal (Fig. 1). However, after 6 days the mitotic figures were uniformly abnormal, with multipolar mitoses and apparently asymmetric segregation of chromatin. Such features are commonly seen in neoplasia. Injection with the control adenovirus did not induce significant cell cycle progression, and the few observed mitoses were morphologically normal (data not shown and see Ref. 20). Furthermore, mitoses observed at 48 h after partial hepatectomy did not display similar abnormalities (data not shown). Thus, protracted cyclin D1 expression (over a matter of days) led to apparent deregulation of the mitotic apparatus, which is a likely precursor of aneuploidy (1–4). Because this suggests a novel mechanism by which cyclin D1 could promote neoplasia, we examined the mitotic and chromosome abnormalities in greater detail.

**Cyclin D1 Triggers Centrosome Amplification and Aberrant Mitotic Spindles in Hepatocytes**—To explore further the rearrangement of hepatocyte mitosis by cyclin D1, we examined the regulation of centrosome number and mitotic spindle structure by using established immunohistochemical techniques. The study of centrosomes and mitotic spindles in fixed tissue specimens is limited by certain considerations (36). We therefore transfected mice with ADV-D1 (or the control vector) for 3 days and then isolated hepatocytes by collagenase perfusion. The cells were plated in culture for 2 days in the presence of epidermal growth factor and insulin, which triggers proliferation of quiescent hepatocytes. Cells were then fixed and co-immunostained with antibodies to centrosome proteins (Cep-135 or γ-tubulin) and α-tubulin, which is the major mitotic spindle protein (4, 25, 26, 36). All γ-tubulin-positive foci also stained with Cep-135 (data not shown), and we therefore elected to use Cep-135 as the marker of centrosomes in hepatocytes because of the quality of the antibody (26).

In normal hepatocytes, or those transfected with the control adenovirus, we observed bipolar mitotic spindles with either two or four centrosomes (Fig. 2A), reflecting the normal population of 2N and binucleated (2N×2N) cells in the liver (37).

![Fig. 1. Cyclin D1 induces abnormal hepatocyte mitoses in vivo.](http://www.jbc.org/)
Similarly, hepatocytes transfected with a recombinant adenovirus encoding human cyclin E (ADV-E) did not demonstrate substantially increased centrosome numbers or abnormal mitotic morphology. In contrast, cyclin D1-transfected hepatocytes uniformly demonstrated irregular multipolar mitotic spindles with supernumerary centrosomes. The number of centrosomes (as defined by distinct Cep-135 or α-tubulin-positive foci) exceeded the number of spindle poles, because several centrosomes sometimes clustered at a single pole (Fig. 2A, inset). As shown in Fig. 2B, cyclin D1-transfected hepatocytes almost always contained more than four centrosomes, and some had ≥20 per cell. Thus, 5 days after cyclin D1 transfection (3 days in vivo and 2 days in culture), hepatocytes demonstrated substantially increased centrosome numbers and markedly abnormal mitotic morphology.

Normal centrosomes consist of a pair of centrioles surrounded by pericentriolar material (5). Centrosomes are typically visualized and quantified by performing immunostaining for pericentriolar proteins such as α-tubulin or Cep135. In Fig. 2C, cells were immunostained with an antibody to centrin (green) and Cep-135 (red) and photographed under high power magnification. D, FACS analysis of propidium iodide-stained hepatocytes. A portion of cyclin D1-transfected cells exhibited DNA contents that did fall into the major 2N, 4N, and 8N peaks. E, the percentage of DAPI-stained cells displaying metaphase morphology under each condition.
Cyclin D1 led to hyperamplification of centrosomes with a normal number of centrioles.

The number of centrosomes per cell has been linked to DNA content, and one potential mechanism of centrosome hyperamplification is abortive cell division that gives rise to polyploid cells with increased centrosome numbers (5, 38). To examine the relationship of cell ploidy to centrosome number, cells were subjected to FACS analysis to examine DNA content (Fig. 2D). Normal or control-transfected hepatocytes demonstrated clear 2N and 4N peaks. Cyclin D1-transfected hepatocytes showed the same peaks, with proportionately more 4N cells and a substantial number of 8N cells. However, a significant number of cells did not fall into the 2N, 4N, or 8N peaks, suggesting that cyclin D1 induced aneuploidy (see below). Furthermore, very few cells with >8N DNA content were observed. In the cyclin D1-transfected cells, centrosome content (Fig. 2B) appeared to be increased to a greater degree than DNA content (Fig. 2D). Thus, persistent cyclin D1 expression may lead to uncoupling of the centrosome cycle from the cell cycle.

Normal hepatocytes proliferate readily in culture in the presence of appropriate growth factors, and previous studies (23, 29, 31) have shown that cyclin D1 is induced in these cells in a mitogen-dependent manner. In Fig. 2E, slides were stained with DAPI, and the number of cells displaying metaphase morphology was found to be similar in cyclin D1-transfected or control cells. Therefore, it is apparent that re-entry into the cell cycle per se was not sufficient to cause the observed centrosome and mitotic abnormalities. Interestingly, cyclin D1 induced the accumulation of supernumerary centrosomes even in interphase cells (Fig. 3), whereas control-transfected or normal cells showed 2–4 centrosomes (data not shown). As was the case for the metaphase cells, the number of centrosomes in cyclin D1-transfected interphase cells did not seem to clearly correlate with the DNA content as assessed by FACS analysis (Fig. 2D). Thus abnormal centrosome numbers were observed in the majority of cyclin D1-transfected cells.

To further examine the relationship between cell ploidy and centrosome number, we studied hepatocytes 2 days following transfection with cyclin D1 (24 h in vivo and 24 h in culture, Fig. 4). At this early time point, the mitotic cells had bipolar spindles after cyclin D1 transfection (Fig. 4A; also see Fig. 1). However, many cells showed a substantially increased number of centrosomes (Fig. 4B), which clustered at the two spindle poles (Fig. 4A). FACS analysis indicated that there was a shift to 4N DNA content and a portion of cells had >4N content (Fig. 4C). However, few cells had 8N content, and the large number of centrosomes seen in some cells (Fig. 4B) was not accompanied by a proportional increase in DNA content. These studies further suggest that cyclin D1 can differentially regulate centrosome number and cell cycle progression. These results also indicate that although short term cyclin D1 overexpression can promote both processes, centrosome overduplication and multipolar spindle formation may be distinctly regulated at early time points.

Cyclin D1 Induces Chromosomal Abnormalities in Hepatocytes—The data shown above indicate that over a period of several days, cyclin D1 induced abnormal mitoses, suggesting the possibility that it may also affect the cellular chromosome content. The FACS data in Figs. 2D and 4C indicated that a substantial portion of cyclin D1-transfected cells contained hyperploid nuclei that did not correspond with the 2N, 4N, or 8N peaks, suggesting that cells with grossly abnormal chromosome content were present. To examine this further, chromosome morphology from control and transfected hepatocytes was evaluated using standard techniques (Fig. 5A). In normal and control-transfected cells, >88% of cells contained the expected 2N, 4N, or 8N chromosome complement. On the other hand, in cyclin D1-transfected cells, only 32% of cells fell within these peaks. The combined data from the FACS analysis and chromosome morphology data indicate that cyclin D1 induces polyplody and the accumulation of cells with grossly abnormal chromosome content that does not fall within the predicted 2N, 4N, and 8N peaks.

To examine further the effect of cyclin D1 on chromosome integrity, mitotic spreads were evaluated for the presence of overt structural abnormalities. As shown in Fig. 5B, cyclin D1 promoted the development of chromosome breaks, free centromeres, and dicentric chromosomes. More than 20% of cyclin D1-transfected hepatocytes demonstrated such abnormalities (Fig. 5C). These data indicate that short term cyclin D1 transfection leads to both numerical and structural chromosome abnormalities in hepatocytes.

The Induction of Mitotic and Chromosome Abnormalities by Cyclin D1 Is Accompanied by Apoptosis—In response to injuries that promote abnormalities of the mitotic spindle or DNA damage, normal cells activate checkpoint mechanisms that inhibit cell cycle progression or induce apoptosis (5, 7, 10). In addition, cells with severe aneuploidy can undergo apoptosis because of “chromosomal insufficiency” (4). To examine whether similar mechanisms were activated following cyclin D1 transfection in our system, we evaluated liver specimens for the presence of apoptosis. As shown in Fig. 6, 6 days following cyclin D1 transfection, a substantial number of hepatocytes were overtly apoptotic as determined by TUNEL staining. These data suggest that the mitotic and chromosome abnormalities induced by cyclin D1 resulted in activation of checkpoint mechanisms that triggered hepatocyte apoptosis.

Protracted Cyclin D1 Expression Leads to Marked Induction of p53 and p21 Gene Expression—In our prior studies, we found that transfection with ADV-D1 led to activation of cyclin-cdk complexes, robust proliferation, and numerous mitotic figures within 1–2 days (20–22). 6 days after transfection, despite continued expression of cyclin D1, cyclin D1-cdk4 and cyclin E-cdk2 complexes were relatively inhibited, and the rates of DNA synthesis and mitosis were relatively diminished compared with 1–2 days (although each of these parameters were still up-regulated compared with normal or control transfected
livers) (20). This suggests that prolonged expression of cyclin D1 induced antiproliferative signals that suppress cyclin/cdk activity and cell cycle progression; our previous data indicated that marked induction of the cdk-inhibitory protein p21 might play a role in this response (20). At the 6-day time point, we speculated that the induction of p21 was due in part to the

**FIG. 4. Centrosome and mitotic spindle morphology at an early time point.** Hepatocytes were transfected for 1 day *in vivo*, placed in culture for 1 day, and analyzed as in Fig. 2. A, immunostaining for Cep135 (red) and α-tubulin (green). The inset shows multiple centrosomes clustering at a single spindle pole. B, centrosome number per mitotic cell. C, FACS analysis.

**FIG. 5. Destabilization of chromosomes by cyclin D1.** Hepatocytes were transfected and harvested as in Fig. 2. After fixation, mitotic spreads were prepared and stained with Giemsa as described under “Experimental Procedures.” A, chromosome number per cell. A total of 50 mitotic spreads were analyzed for each condition. B, representative micrographs of chromosome abnormalities in cyclin D1-transfected cells. C, quantification of the number of chromosome abnormalities per mitotic cell.
activation of checkpoint mechanisms that respond to chromosome and/or mitotic spindle abnormalities. Studies in other systems would predict that p53 may be involved in these checkpoints and can induce both p21 expression and apoptosis (7, 10). To examine the potential involvement of p21 and p53 in our system, we evaluated the expression of these genes by RT-PCR (Fig. 7). As shown previously, cyclin D1 induced p21 1 day after transfection, presumably as part of the normal cell cycle program. 6 days after transfection, despite diminished cyclin D1/cdk4 activity (20), p21 mRNA was induced to much higher levels. Furthermore, p53 mRNA was also markedly induced at 6 days. These studies suggest that the mitotic and chromosome abnormalities induced by protracted cyclin D1 expression may promote checkpoint mechanisms involving p53 and p21.

Previous studies have documented that p21 plays a role in regulating centrosome number in response to other stimuli, presumably by modulating cdk activity (5, 7, 10). To determine whether p21 similarly regulated the development of supernumerary centrosomes following cyclin D1 expression, we examined the response in p21−/− mice. By using the conditions outlined in Fig. 2, cyclin D1 induced an average of 14.63 ± 4.75 centrosomes in hepatocytes isolated from p21−/− mice as compared with 8.48 ± 3.63 centrosomes in matched wild-type mice. This suggests that the marked induction of p21 in response to cyclin D1 overexpression moderately diminishes the accumulation of supernumerary centrosomes.

Centrosome Abnormalities Persist for Months after Cyclin D1 Transfection—Our data indicate that the induction of mitotic and chromosome abnormalities by cyclin D1 is associated with both a cell cycle arrest and apoptosis. This suggests that the hepatocytes activated normal checkpoint mechanisms that could result in the elimination of abnormal cells. Previous studies have shown that first-generation recombinant adenoviruses, such as those used in our studies, promote gene expression that lasts several weeks. In our previous studies, we found that cyclin D1 expression was no longer detectable 1 month following transfection (20). To examine whether centrosome abnormalities persisted after cyclin D1 was no longer expressed, we examined hepatocytes 4 months after cyclin D1 transfection. As shown in Fig. 8, normal or control-transfected mice contained 2–4 centrosomes, in a pattern similar to that seen in younger mice (Fig. 2). On the other hand, 13% of cyclin D1-transfected hepatocytes contained >4 centrosomes. Thus, no hepatocytes with massively increased centrosome number (as seen at early time points after transfection) were observed in the mice 4 months after transfection. This suggests that the most abnormal cells were eliminated by the activation of normal checkpoint mechanisms. However, a portion of cells retained abnormal centrosome number, indicating that transient overexpression of cyclin D1 led to long lasting changes in the mitotic apparatus in some cells.

Cyclin D1 Induces Centrosome and Mitotic Spindle Abnormalities in Breast Epithelial Cells—The studies described above suggest that persistent expression of cyclin D1 promotes accumulation of supernumerary centrosomes and aneuploidy in hepatocytes. To determine whether cyclin D1 might have a similar effect in other cells, we transduced human mammary epithelial cells with the cyclin D1 or control adenoviruses, placed in culture for 3 days, and immunostained for Cep-135 as in Fig. 2. Hepatocytes were also isolated from age-equivalent untransfected normal mice. The number of centrosomes per mitotic cell is shown.
The mitotic apparatus were induced early during the oncogenic development of frank malignancy, suggesting that abnormalities of transgenic mice with hepatic cyclin D1 expression were noted widespread random aneuploidy that underlies the "aneuploidy-suggest that overexpression of cyclin D1 may predispose to abnormalities during the process of carcinogenesis (1, 2, 42). In organs, which leads to the development of cancer after a delay of many months (12, 13, 40). This suggests that over time, genome rearrangements and amplification (12). Indirect evidence that genomic instability, because the most clearly defined role of cyclin D1 is activation of the cdk4 and cdk6 kinases in late G1 phase, which results in phosphorylation of retinoblastoma protein and transcription of E2F-dependent genes required for S phase (12). The cyclin D1-cdk4 complex also sequesters cdk inhibitors such as p21 or p27, thereby promoting activation of cyclin-cdk complexes acting downstream in the cell cycle. Cyclin D1 has been implicated in other cellular processes including transcriptional control, ribosome biogenesis, cell growth, differentiation, apoptosis, adhesion, and motility (12, 43). In addition, clinical studies suggest that overexpression of cyclin D1 is associated with chromosome abnormalities in several different cell types (15–18). However, it is difficult to determine from these clinical studies whether cyclin D1 overexpression is a cause or a result of genomic instability, because the cyclin D1 locus is prone to rearrangements and amplification (12). Indirect evidence that cyclin D1 can cause genetic alterations comes from transgenic mice with constitutive expression of this protein in various organs, which leads to the development of cancer after a delay of many months (12, 13, 40). This suggests that over time, persistent cyclin D1 expression may contribute to the "mutator phenotype" that results in the acquisition of specific genetic abnormalities during the process of carcinogenesis (1, 2, 42). In addition, the marked mitotic abnormalities seen in this study suggest that overexpression of cyclin D1 may predispose to widespread random aneuploidy that underlies the "aneuploidy-cancer theory," which holds that malignancy is induced by the abnormal dosage of a large number of genes (44). Notably, transgenic mice with hepatic cyclin D1 expression were noted to have aberrant hepatocyte mitotic figures prior to the development of frank malignancy, suggesting that abnormalities of the mitotic apparatus were induced early during the oncogenic process (40). However, to our knowledge, previous experimental studies have not found that cyclin D1 overexpression induces centrosome aberrations and chromosome instability.

Although genetic studies have not unequivocally demonstrated that alterations in centrosome numbers cause aneuploidy, centrosome abnormalities are observed in a wide variety of human malignancies and have been correlated with chromosome instability (reviewed in Refs. 3–6). The factors that promote centrosome accumulation in malignant cells have not been defined, but several mechanisms have been proposed (5, 10, 38). One possibility is that cells fail to undergo cytokinesis, leading to both polyploidy and accumulation of extra centrosomes. According to this model, an increased number of centrosomes should be linked to chromosome content. A second possibility is that the centrosome duplication cycle becomes uncoupled from DNA replication. Other potential mechanisms include cell fusion and the de novo formation of centrosomes. These possibilities are not necessarily mutually exclusive and each may be operative under certain circumstances (5). Regardless of the mechanisms involved, centrosome aberrations are likely to contribute to chromosome instability in human tumors (3–6).

Normal hepatocytes have been shown to regulate mitosis distinctly from typical models (37). As the animal ages, increasing numbers of binucleated cells and polyploid nuclei (4N and 8N) are observed. Binucleated hepatocytes appear to result from a failure of cytokinesis at the end of mitosis (37). At the time of cell division, binucleated cells contain four centrosomes that cluster into two spindle poles, resulting in a bipolar spindle apparatus that produces two 4N cells (an example of this is shown in the control-transfected cell in Fig. 4A). In the current studies, we found that at early time points after cyclin D1 transfection, hepatocytes displayed bipolar mitotic spindles despite overduplication of centrosomes (Figs. 1 and 4). Thus, like normal binucleated hepatocytes, extra centrosomes cluster at two spindle poles during the initial phase of cyclin D1 overexpression. This suggests that spindle pole formation is regulated distinctly from centrosome number, as suggested in other systems (5, 37). However, at 6 days, when centrosome number was greatly increased, cells consistently displayed multipolar mitoses as well as chromosome abnormalities. We also found that the increase in centrosome number triggered by cyclin D1 did not clearly parallel the corresponding increase in DNA content. The rapid induction of centrosome overduplication (within 48 h), along with the observation that centrosome number did not seem closely linked to ploidy, suggests that the abnormalities were not simply a result of failure of cytokinesis and polyploidy. The data suggest that persistent expression of cyclin D1 can promote centrosome duplication out of proportion to cell cycle progression. However, additional studies will be required to unravel the relationship between cyclin D1, cell cycle progression, centrosome number, spindle pole formation, and aneuploidy.

This study did not directly address the mechanism(s) by which cyclin D1 may regulate centrosome accumulation. Cyclin D1 transfection induces cell cycle progression and downstream cell cycle mediators including cyclin E/cdk2 and cyclin A/cdk2 in hepatocytes (20, 23). These complexes are known to promote centrosome duplication and phosphorylation of several different centrosome proteins (5, 8, 9). Thus, cyclin D1 may act by promoting activation of cdk2, which in turn induces centrosome and mitotic spindle abnormalities, resulting in aneuploidy. Interestingly, transfection with cyclin E alone did not promote marked centrosome abnormalities in our system. This may be due to the fact that cyclin E transfection does not induce activation of cyclin E/cdk2 or cell cycle progression in hepatocytes, presumably because these cells contain sufficient monomeric proteins for competent cell cycle progression.
p27 to inhibit these complexes (19, 45). Further studies will be required to determine whether cyclin D1 acts directly on centrosome targets or whether it mediates effects by activating downstream cell cycle genes.

Our results differ from a previous report indicating that permanent transfection of cyclin E, but not cyclin D1, induces aneuploidy in cultured cells (46). The discrepancy may be due to differences in transfection techniques, experimental conditions, or susceptibility of various cell types. Our finding that short term cyclin D1 overexpression induced centrosome and mitotic spindle abnormalities in breast epithelial cells (Fig. 9) indicates that this effect is not limited to hepatocytes and could potentially affect chromosome stability in different types of tumors with constitutive expression of this protein. Indeed, several reports have shown that cyclin D1 is associated with aneuploidy in a variety of malignant cell types (15–18), which is consistent with the notion that persistent expression of this protein promotes mitotic abnormalities in human malignancies.

The mice transiently transfected with cyclin D1 mice did not develop overt signs of malignancy during the relatively short period of observation. However, this was not formally addressed because we did not sustain a large number of animals over a longer period. The striking mitotic abnormalities seen 6 days after cyclin D1 transfection were associated with substantial hepatocyte apoptosis as well as induction of p53 and p21 in these livers. Previous studies in p53 and p21 knockout cells indicate that these proteins play an important role in preventing centrosome abnormalities, presumably by activating checkpoint mechanisms and inhibiting cdk2, respectively (10). In our studies, centrosome and chromosome abnormalities apparently induced checkpoint mechanisms that promoted apoptosis and cell cycle arrest, which effectively removed the most aberrant cells. Interestingly, we did note that a proportion of cells demonstrated increased numbers of centrosomes 4 months after cyclin D1 transfection. Because the first generation adenovirus system produces transgene expression that lasts only weeks (20, 24), these results indicate that some of the centrosome changes induced by cyclin D1 overexpression may persist even when the protein is no longer expressed. In the setting of persistent hepatocyte proliferation or mutations of p53 gene (which are commonly observed in hepatocellular carcinoma (HCC) (41, 47, 48)), these cyclin D1-induced centrosome changes could play a potentially important role in the development of aneuploidy.

In humans, HCC occurs almost exclusively in the setting of longstanding chronic liver diseases, which generally demonstrate ongoing hepatocyte destruction and proliferation (41, 48). Previous studies (49–51) have found that a high level of hepatocyte proliferation observed on liver biopsy specimens correlates with an increased risk of HCC. Both HCC and preneoplastic foci of dysplastic hepatocytes are characterized by widespread and heterogeneous chromosome changes (41, 47). Although it is absent in normal adult liver, cyclin D1 is upregulated following liver injury in diverse animal models and is expressed in human liver demonstrating evidence of hepatocyte replication (31). Clinical HCC specimens with high level cyclin D1 expression were found to have a highly dysplastic morphologic phenotype (52), although the relationship of cyclin D1 expression and chromosome abnormalities has not been formally evaluated in this cancer (to our knowledge). The current study suggests that persistent cyclin D1 expression induced by chronic mitogenic stimulation, oncogenic mutations of upstream signaling molecules, or chromosome rearrangements involving the cyclin D1 gene could potentially contribute to the striking and apparently random chromosome abnormalities seen in many HCCs (41, 47).

These findings should raise a note of caution regarding recombinant adenoviruses, which have been used extensively to achieve high efficiency transient transfection in culture and in vivo (24). These vectors readily transfect hepatocytes in the liver after intravenous injection and can target genes to other organs as well (24). Recombinant adenoviruses expressing cyclin D1 trigger proliferation of quiescent hepatocytes and cardiomyocytes in vivo, suggesting a possible strategy to promote adaptive parenchymal cell replication in disease states (20, 53). The carcinogenic potential of adenoviral vectors has been thought to be minimal because they do not insert into the chromosome DNA. National Institutes of Health guidelines suggest that Biosafety Level II containment measures are sufficient for recombinant adenoviruses. However, our findings indicate that even short term overexpression of cyclin D1 produces chromosome instability. Thus, strategies to transiently transfect cyclin D1 (and conceivably other growth-promoting genes) could induce unanticipated genetic alterations even if the vectors do not integrate into cellular DNA. More stringent laboratory containment procedures for viral vectors encoding potential oncogenes may therefore be advisable.

The results presented here indicate that aberrant cyclin D1 expression rapidly induces alterations of the mitotic apparatus and aneuploidy in normal cells. We believe that these studies provide the most direct evidence to date that overexpression of cyclin D1 alone can lead to genomic instability. Furthermore, our short term experiments showed that under these conditions, centrosome duplication was not proportional to DNA replication, suggesting that the centrosome cycle and the cell cycle can be uncoupled. Cyclin D1 should therefore be added to the list of cancer-related genes that regulate centrosome duplication and the mitotic spindle apparatus (5). The induction of chromosome instability by cyclin D1 could play an important role in the carcinogenic effect of this oncogene.

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Christopher J. Nelsen, Ryoko Kuriyama, Betsy Hirsch, Vivian C. Negron, Wilma L. Lingle, Melissa M. Goggin, Michael W. Stanley and Jeffrey H. Albrecht

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