Repression of cyclin D1 as a target for germ cell tumors

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Abstract. Metastatic germ cell tumors (GCT) are curable, however GCTs refractory to cisplatin-based chemotherapy have a poor prognosis. This study explores D-type cyclins as molecular targets in GCTs because all-*trans*-retinoic acid (RA)-mediated differentiation of the human embryonal carcinoma (EC) cell line NT2/D1 is associated with G1 cell cycle arrest and proteasomal degradation of cyclin D1. RA effects on D-type cyclins are compared in human EC cells that are RA sensitive or dually RA and cisplatin resistant (NT2/D1-R1) and in clinical GCTs that have both EC and mature teratoma components. Notably, GCT differentiation was associated with reduced cyclin D1 but increased cyclin D3 expression. RA was shown here to repress cyclin D1 through a transcriptional mechanism in addition to causing its degradation. The siRNA-mediated repression of individual cyclin D species resulted in growth inhibition in both RA sensitive and resistant EC cells. Only repression of cyclin D1 occurred *in vitro* and when clinical GCTs mature, implicating cyclin D1 as a molecular therapeutic target. To confirm this, the EGFR-tyrosine kinase inhibitor, Erlotinib, was used to repress cyclin D1. This inhibited proliferation in RA and cisplatin sensitive and resistant EC cells. Taken together, these findings implicate cyclin D1 targeting agents for the treatment of GCTs.

Introduction

Cyclin-dependent kinases (cdks) play a major role in ordered cell cycle progression and their activity is regulated in a cell cycle-specific manner by interacting with cyclins, as reviewed (1). Cyclin D species interact with cdk4 and cdk6. A major substrate of these complexes is the retinoblastoma (Rb) protein. Rb is maintained in a hypophosphorylated state through most of the G1 phase, becoming hyperphosphorylated in late G1, resulting in release of E2F transcription factors, which in turn augments expression of genes required for S phase (2). A further layer of control preventing entry into S phase exists in the form of cdk inhibitors (CKIs) (3). Thus, the 'Rb pathway' represented by cyclin D/cdk4/CKI/Rb constitutes a tightly controlled G1/S checkpoint, the importance of which is highlighted by clinical findings that one component of the pathway is mutated or deregulated in the majority of human cancers (4).

Germ cell tumors (GCTs) are the most common carcinomas of men between the age of 15 and 35. Cure of GCTs is possible with cisplatin-based chemotherapy, even in disseminated disease, however, patients refractory to treatment do exist and innovative strategies for the treatment of these cases are needed. GCTs can exhibit mature teratoma formation, indicating *in vivo* differentiation potential of these tumors. The exquisite sensitivity of GCTs to chemotherapeutic agents, spontaneous differentiation patterns and characteristic chromosomal abnormalities make them invaluable models for solid tumor biology studies (5). In this study, the human embryonal carcinoma (EC) cell line NT2/D1 was used to explore retinoid mediated cell cycle arrest and differentiation mechanisms. The NT2/D1 line is an attractive model for the study of GCTs because: a) it carries the isochromosome 12p marker diagnostic of GCTs (6), b) it exhibits unique immunophenotypic markers for the differentiated and undifferentiated states (7,8) and c) it is pluripotent, differentiating into a neuronal phenotype and other lineages after RA-treatment (9,10). The pluripotent nature of NT2/D1 cells provides a valuable and practical complement to human embryonic stem cells as an *in vitro* model of early human development (5,11).

We previously reported that cyclin D1 protein decreases in NT2/D1 cells after RA-treatment (12). This is associated with increased cyclin D1 ubiquitination that precedes induction of differentiation. With this decline, RA-treatment also arrests cells in the G1 phase of the cycle, blocking further rounds of DNA synthesis. The RA-resistant NT2/D1-R1 line,

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does not demonstrate growth arrest or accumulate in G1, and has persistent cyclin D1 over-expression, despite RA-treatment (12). This strongly implicates a link between cyclin D1 levels and retinoid-mediated growth suppression.

Engineered over-expression of a degradation-resistant cyclin D1 species in NT2/D1 cells delays RA-induced differentiation and cell cycle arrest (12). The effects of RA-treatment on D-type cyclins have not been explored comprehensively in ECs or GCTs. Of the members of the D-type cyclin family (cyclin D1, cyclin D2 and cyclin D3) cyclin D1 is the most studied with over-expression documented in diverse tumors including breast (13-15), parathyroid adenomas (16), and mantle cell lymphomas (17,18). Cyclin D2 is amplified and over-expressed in subsets of GCTs (19,20). This is thought to arise from its location on chromosome 12p. Recently, the critical region of 12p over-represented in male GCTs was mapped to a 1750-3000 kb region, which did not include the cyclin D2 locus (21). Cyclin D2 has been proposed as involved in GCT development and progression, but not in response to chemotherapeutic or differentiation agents (20,22). Cyclin D3 has been studied in male GCTs (23) where an increase in cyclin D3 levels was noted during differentiation. Cyclin D3 is elevated at the onset of myogenic terminal differentiation (24). D-type cyclins differ in their cell tissue context distribution. Cyclin D1 has been the focus of several studies describing cdk4-independent properties, including physical association with transcription factors and coactivators, as reviewed (25). Distinct functional roles for the different D-type cyclins likely exist, but redundant effects have also been noted in D-type cyclin knockout models, as reviewed (26).

In this study, all three cyclin D species were examined to compare their expression profiles in differentiation sensitive as compared to resistant EC cells, as well as in clinical GCTs. Selective siRNA-mediated repression of individual D-type cyclins was used to learn if these cyclins had different effects on EC growth and differentiation. Using paraffin-embedded sections of ECs that also contained mature teratoma, immunohistochemical expression profiles for Ki-67, and D-type cyclins were examined. Findings presented here reveal that repression of any cyclin D species is sufficient to arrest growth of GCTs, but it is cyclin D1 that is preferentially associated with differentiation-dependent growth arrest in cultured EC cells and in clinical GCTs. Indeed, an inverse relationship between cyclin D1 and cyclin D3 expression was found with GCT differentiation. Taken together, these findings indicate that pharmacological agents that target cyclin D1 for repression should be considered for GCT therapy.

Materials and methods

Cell culture conditions. NT2/D1 and NT2/D1-R1 human EC cells were cultured in high glucose DMEM (Invitrogen, CA) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM/l glutamine, and 10% fetal bovine serum at 37˚C under humidified 5% CO_2. For stable expression of luciferase-expressing constructs, cells were plated at $2x10^5$ cells/well in 6-well tissue culture plates and were cotransfected with 2.0 μg of luciferase reporter plasmid DNA and 0.5 μg of pcDNA3.1 (Invitrogen) using a modified calcium phosphate precipitation technique (12). Cells were exposed to DNA precipitate for 14-16 h, then washed and trypsinized for transfer to 10 cm tissue culture plates and treatment with G418 (500 μg/ml). G418 resistant colonies were harvested for further analyses. Protein concentrations were determined using the Bradford assay (Bio-Rad, CA) and luciferase activity was measured using the Promega (WI) luciferase assay system. Erlotinib was dissolved in the vehicle dimethyl sulfoxide (DMSO) at 10 mM. Cells were plated at $1x10^5$ (NT2/D1) and $0.5x10^4$ (NT2/D1-R1) per well in 6-well plates in triplicate. Erlotinib was added 24 h after cell plating and fresh drug and media were added after 48 h. Cells were trypsinised and harvested at 72 h and counted using a hemacytometer.

RA induction protocol. Western and Northern blot analyses. RA was dissolved in DMSO as a 10 mM stock solution and stored in liquid nitrogen. Cells were treated with 10 μM RA. Total cellular RNA was isolated using TriReagent (Molecular Research Center, OH). RNA was size-fractionated on a 1% agarose denaturing gel before transfer to a nylon membrane (Schleicher and Schuell, NH). Northern filters were hybridized with probes random-labeled with [^{32}P]-dCTP at $1x10^6$ cpm/ml in hybridization solution [0.5 M sodium phosphate, pH 7.0, 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin, and 1 mM EDTA] at 67˚C overnight. Filters were stringently washed, as previously described (27). The cDNA probes used for Northern blot analyses were respectively a 1.1 kb *Hin*d III restriction endonuclease fragment isolated from the pRcCMV-cyclin D1-HA vector, a 1.0 kb *Hin*d III/*Xba* I restriction endonuclease fragment isolated from the pRcCMV-cyclin D2 vector and a 1.1 kb *Hin*d III/*Xba* I restriction endonuclease fragment isolated from the pRcCMV-cyclin D3-HA vector.

Cells were lyzed in a modified radioimmune precipitation buffer, as previously described (28). Total protein lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Antibodies used individually recognized cyclin D1 (M20), cyclin D2 (C17), cyclin D3 (C16) or actin (C11) (Santa Cruz, CA). Primary antibodies were detected with horseradish peroxidase conjugated secondary antibodies (Santa Cruz and Amersham, IL) and visualized using an enhanced chemiluminescence system (Amersham).

Heterogeneous nuclear RNA analysis. Total cellular RNA was isolated as already described and any contaminating DNA was removed using the DNA*free* system (Ambion, Austin, TX). Reverse transcription (RT) of RNA was performed using a cDNA synthesis system (Invitrogen). Polymerase chain reaction (PCR) assays were performed using *Taq* polymerase (Invitrogen) according to the manufacturer's instructions. Cycle number was optimized for each primer pair. The primer pairs used were: forward primer cyclin D1-exon1-2 5'-TGTGCTGCGAAGTGGAAACC-3' and reverse primer cyclin D1-intron1-2 5'-CAAGTTGCAGGGAAGTCT TAAG-3'; ß-actin forward primer 5'-GCGGGAAATCGTGC GTGACA-3' and ß-actin reverse primer 5'-AAGGAAGGC TGGAAGAGTGC-3'; and RAR-ß forward primer 5'-GAGC GATCCGAGCAGGGTTT-3' and RAR-ß reverse primer 5'-CTGGCAGACGAAGCAGGGTTT-3'.

siRNA repression of D-type cyclins. Double-stranded siRNA with 19-nucleotide duplex RNA and a 2-nucleotide deoxythymidine overhang at the 3' region were synthesized (Dharmacon, CO and Qiagen, CA). Two different siRNAs were designed to target independently human cyclin D1, cyclin D2 or cyclin D3 mRNAs. The sequences chosen were cyclin D1-1 5'-CACCAGCTCCTGTGCTGCG-3', cyclin D1-2 5'-CAAACAGATCATCCGCAAA-3', cyclin D2-1 5'-GTGC GTGCAGAAGGACATC-3', cyclin D2-2 5'-TAGCCTGCA GCAGTACCGT-3', cyclin D3-1 5'-GATGCTGGCTTACTG GATG-3' and cyclin D3-2 5'-GATGGCTCCTCTCAGT ACT-3'. Firefly luciferase pGL2 siRNA (5'-CGTACGCGGA ATACTTCGA-3') and scrambled II (scb) (5'-GCGCGCTTT GTAGGATTCG-3') (Dharmacon) served as siRNA duplex controls. Transfection of siRNA was performed with Oligofectamine (Invitrogen), as previously described (29). To assess effects on cell growth, EC cells were trypsinized 24 h after transfection and plated at $5x10^4$ cells per well in 6-well plates. Triplicate wells were counted for each time-point post-transfection. Results were expressed as mean ± standard deviation for a representative experiment.

Differentiation marker studies. Indirect fluorescence-activated cell sorter (FACS) analysis to evaluate RA-induced neuronal differentiation of NT2/D1 cells was performed using established techniques (12). Briefly, NT2/D1 cells were harvested by trypsinization and incubated with monoclonal antibodies to established EC differentiation markers. The A2B5 antibody was isolated from a hybridoma culture and recognizes a neuronal epitope in RA-treated NT2/D1 cells (ATCC). SSEA-3 (Developmental Studies Hybridoma Bank, University of Iowa, USA) recognizes an embryonal antigen, which is lost as EC cells differentiate (8). Cells were indirectly assayed with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody, and fluorescence was measured, as described (12).

Immunohistochemistry. Clinical GCT specimens were fixed and embedded in paraffin using established techniques previously described (30). The immunohistochemical markers examined were: cyclin D1, cyclin D3 and Ki-67. Antibodies used to detect these species were: cyclin D1 (CP236A, Biocare Medical, CA), cyclin D3 (C17 Santa Cruz) and Ki-67 (clone MIB1, Dako, Glostrup, Denmark). Use of these clinical GCT specimens was approved by the Dartmouth College Institutional Review Board (IRB).

Results

Expression of D-type cyclin expression in retinoid sensitive and resistant GCT cells. Cyclin D1 protein levels are substantially repressed after RA-treatment of NT2/D1 cells, but decline less in RA-resistant NT2/D1-R1 cells (Fig. 1A). Basal levels of cyclin D1 protein were much higher in the RA resistant than sensitive EC cells. Cyclin D2 protein levels did not appreciably change after RA-treatment or increased slightly and were similar in both retinoid sensitive and resistant EC cells. Conversely, cyclin D3 protein levels increased markedly with RA-treatment of NT2/D1 cells, but this was much less evident in NT2/D1-R1 cells. Basal cyclin D3 protein expression

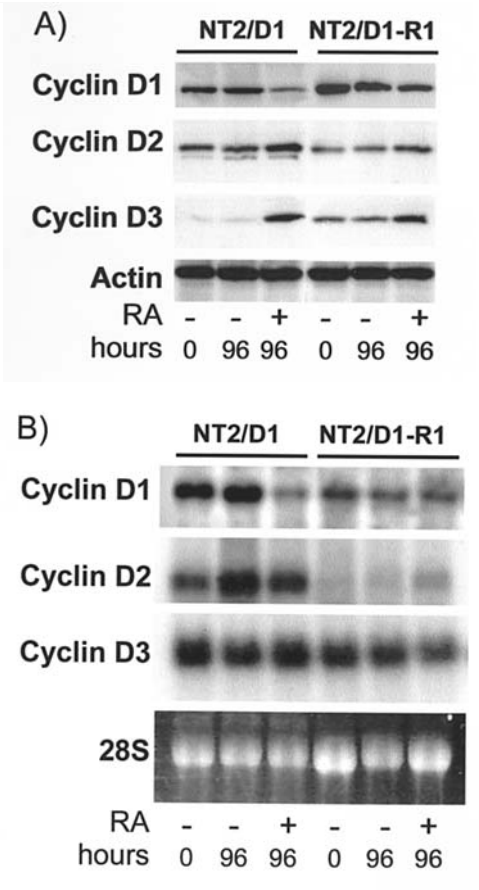

Figure 1. Northern and Western blot analyses of cyclin D species before and after RA-treatment of the human EC cell line NT2/D1 and the RA-resistant cell line, NT2/D1-R1. (A) Western blot analyses of D-type cyclin species normalized to total 28S ribosomal RNA. (B) Northern blot analyses of D-type cyclin species normalized to ß-actin expression.

was also elevated in the retinoid resistant as compared to the sensitive EC cells.

Northern blot analyses of D-type cyclins revealed that after 96 h of RA-treatment, cyclin D1 mRNA levels were repressed in NT2/D1 cells (Fig. 1B). This was not observed in NT2/D1-R1 cells where there was no appreciable change in cyclin D1 mRNA levels and basal levels were lower than seen in NT2/D1 cells. Appreciable changes in cyclin D2 mRNAs were not seen in either cell line, although the basal level of cyclin D2 mRNA was much lower in NT2/D1-R1 than NT2/D1 cells. Cyclin D3 mRNA expression was similar over the same time course for both cell lines. Comparing immunoblot and Northern blot analyses for D-type cyclins between NT2/D1 and NT2/D1-R1 cells established a markedly different pattern between them. NT2/D1-R1 cells have equal or lower levels of mRNA for each D-type cyclin, but exhibit equal or higher protein expression levels. This is most likely due to increased cyclin D stability in NT2/D1-R1 cells; the half-life of cyclin D1 is higher in these cells than in the NT2/D1 cells (data not shown). Prior work by others indicates that cyclin D3 protein stability can increase when it is bound to the cell cycle inhibitor p27 (31-34) although other researchers did not find this to be the case (35). The expression of p27 protein increases with RA-treatment of NT2/D1 cells (34,35; data not shown).

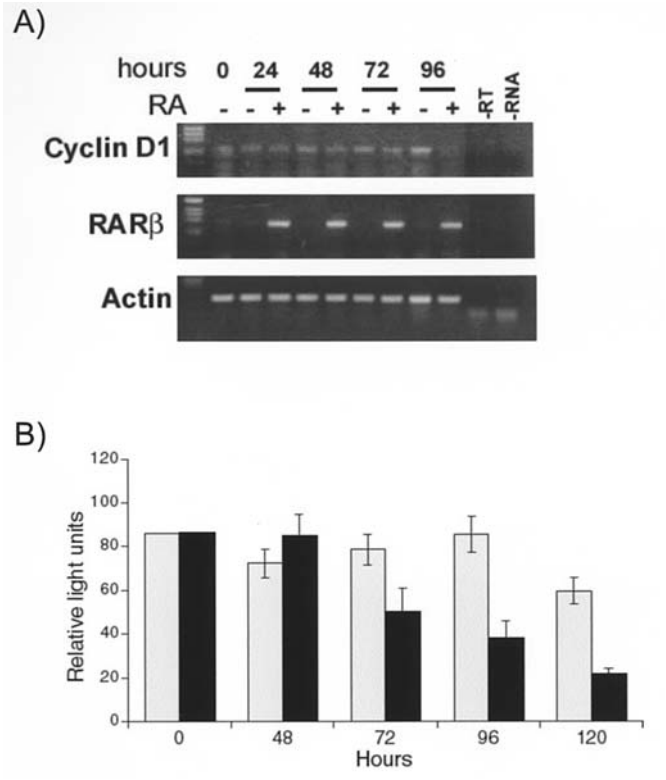

Figure 2. RA-treatment of NT2/D1 cells reduces the rate of cyclin D1 transcription. (A) Hetero-nuclear polymerase chain reaction (hnPCR) assay of cyclin D1. The cDNA levels were normalized to ß-actin mRNA levels. RAR-ß PCR demonstrates the expected response to RA-treatment of a known RA target gene. (B) Cyclin D1 promoter activity measured following RA-treatment in NT2/D1 cells with a stably integrated cyclin D1 promoter-luciferase construct. Each luciferase reading was made in triplicate and error bars represent standard deviation. Shaded bars represent RA treatment and open bars represent vehicle control. These data are representative results from at least three independent experiments.

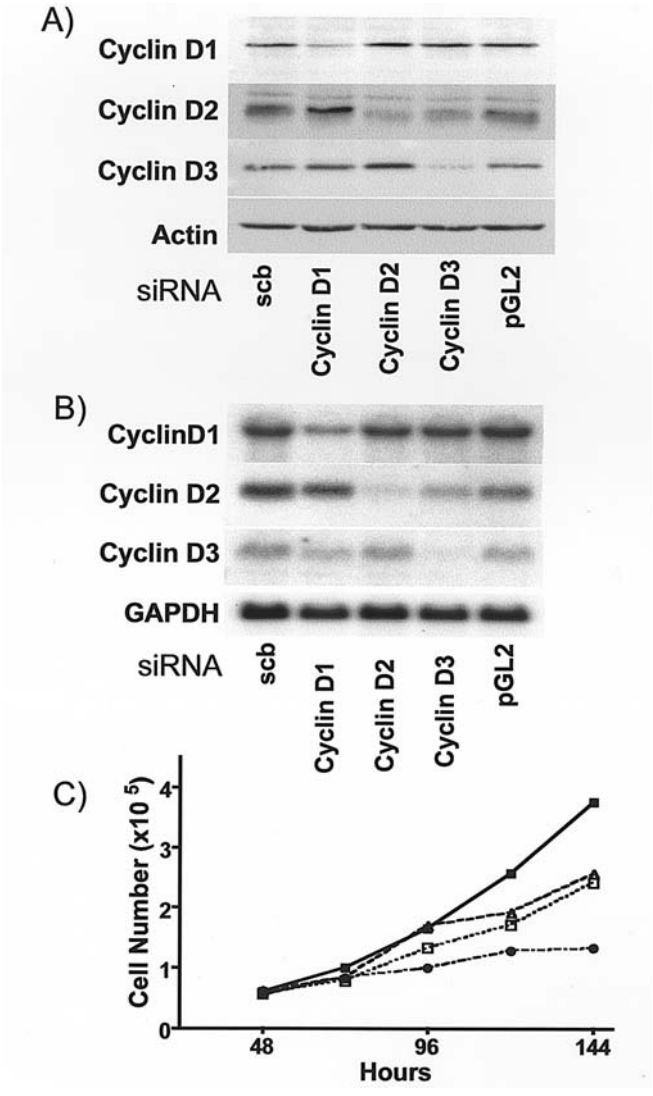

Figure 3. The effects of siRNA-mediated repression of individual cyclin D-type species in RA sensitive NT2/D1 cells. (A) Western blot analyses of D-type cyclins following siRNA treatments specific to individual cyclin D species in NT2/D1 cells. (B) Northern blot analyses of D-type cyclins after siRNA treatment specific to individual cyclin D species in NT2/D1 cells. (C) Cell proliferation analyses of NT2/D1 cells after siRNA treatments. The siRNAs used were: scrambled (scb) control (■), cyclin D1-1 (❑), cyclin D2-1 (▲), and cyclin D3-1 (●).

Retinoid repression of cyclin D1 mRNA. Although initially due to an acceleration of cyclin D1 proteasome degradation (12), longer RA-mediated repression of cyclin D1 is also due to a decrease in cyclin D1 mRNA expression, as shown in Fig. 1B. Cyclin D1 mRNA stability did not decrease with RA-treatment (data not shown). Using hetero-nuclear PCR (hnPCR) assays the levels of prespliced cyclin D1 mRNA were found to decrease between 72 and 96 h of RA-treatment (Fig. 2A). This indicated that the rate of cyclin D1 transcription had decreased. Of the three cyclin D species more is known about the control of cyclin D1 transcription in part because this D-type cyclin is frequently over-expressed in human carcinogenesis, as reviewed (25). The cyclin D1 promoter contains multiple domains that respond to oncogenic signals, as reviewed (25,36). Initial studies with a 1.7 kb region of the human cyclin D1 promoter (37) reporter construct did not reveal repression of promoter activity with RA in NT2/D1 cells (data not shown). These experiments were performed by transiently transfecting this reporter plasmid into NT2/D1 cells, treating with RA for varying time periods and then assaying for luciferase activity. However, with stable integration of the cyclin D1 promoter-luciferase construct into NT2/D1 cells the RA-mediated repression of the cyclin D1 promoter was evident, as shown in Fig. 2B. Pools of G418-resistant NT2/D1 cells were treated with RA or the vehicle (DMSO) and the luciferase activities were normalized to protein concentration for each time-point. After 72 h of RA-treatment a significant repression of cyclin D1 promoter activity was detected. This declined further after 96 and 120 h. A RSV promoter-luciferase construct was also used to generate a stable pool of NT2/D1 cells. Treatment of these cells with RA did not result in repression of luciferase activity as seen with the cyclin D1 promoter (data not shown).

Repression of D-type cyclins by siRNAs. To determine if repression of the individual D-type cyclins had growth or differentiation-inducing effects on EC cells, independent siRNA duplexes were designed to target each of them. Fig. 3A and B show respectively Western and Northern blot analyses of D-type cyclins in response to siRNA transfection. The siRNAs cyclin D1-1 and cyclin D2-1 specifically repressed only the desired species, as indicated in this figure and cyclin

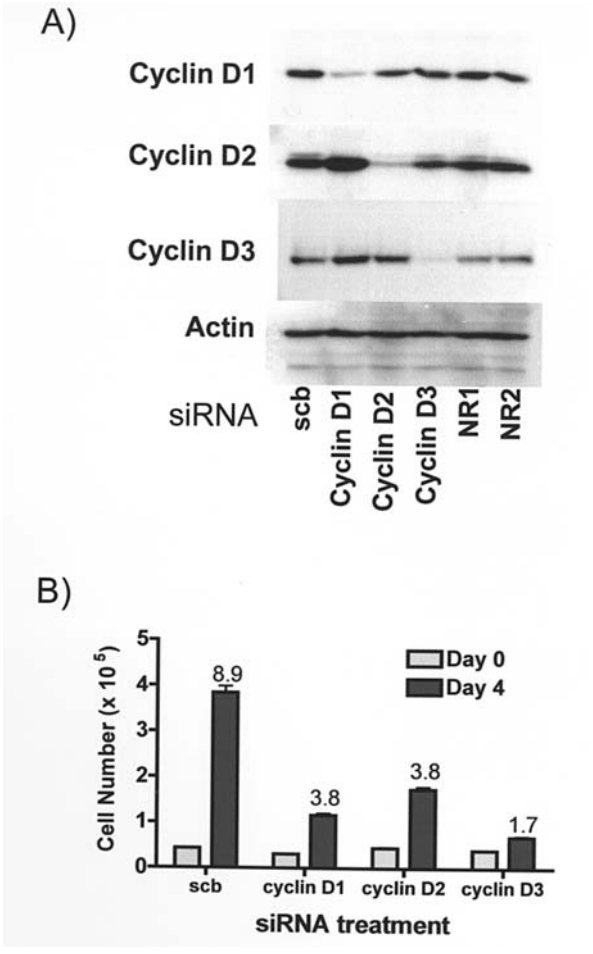

Figure 4. The effects of siRNA-mediated repression of individual cyclin D-type species in RA resistant NT2/D1-R1 cells. (A) Western blot analyses of D-type cyclins after siRNA treatments specific to individual cyclin D species in RA resistant NT2/D1-R1 cells. (B) Cell proliferation analyses of NT2/D1-R1 cells post-siRNA treatment. Numbers over the bars indicate fold increase in cell number over the 4 days. Each cell count was made in triplicate and error bars represent standard deviation. The data are representative of at least three separate experiments. NR1 is pGL2 and NR2 is Scb2 siRNA.

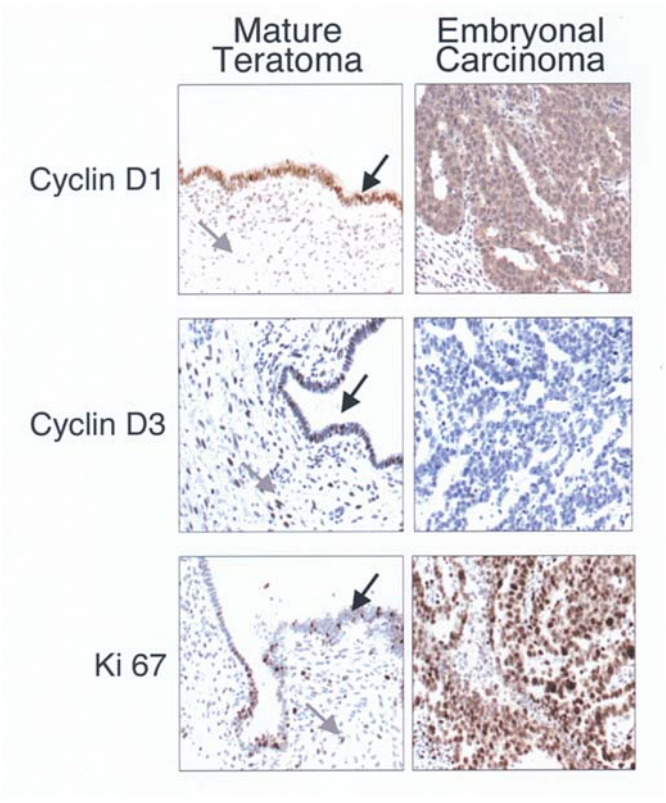

Figure 5. A representative clinical GCT exhibiting both embryonal carcinoma (EC) and mature teratomatous elements. This case was independently examined for immunohistochemical expression of cyclin D1, cyclin D3 and Ki-67. These analyses revealed an inverse relationship between cyclin D1 and cyclin D3. Black arrow indicates a more proliferative region of teratoma and the gray arrow indicates a less proliferative region of teratoma based on Ki-67 immunostaining. There is abundant expression of cyclin D1 and repressed expression of cyclin D3 in EC GCT components. In contrast, cyclin D3 expression is increased in mature teratomatous GCT components.

D3-1 siRNA substantially repressed cyclin D3 expression, with a minimal repression of cyclin D2 levels also detected. In proliferation assays, repression of each cyclin D species also inhibited EC cell growth. The most evident repression was the siRNA directed against cyclin D3 and this is likely due to this siRNA duplex repressing both cyclin D3 and cyclin D2. Cell cycle distribution analyses showed, as expected, a consistent increase in G1-phase cells after siRNA-mediated repression of each cyclin D species and bromodeoxyuridine incorporation analyses revealed a decrease in cells actively synthesizing DNA (data not shown). Experiments using the independent cyclin D siRNAs (cyclin D1-2, cyclin D2-2 and cyclin D3-2) confirmed effects on growth seen with the other D-type cyclin siRNAs (data not shown),

These D-type cyclin targeting-experiments were extended from RA sensitive to resistant EC cells. The siRNA-mediated repression of cyclin D species in the NT2/D1-R1 cells exhibited results similar to those observed in NT2/D1 cells (Fig. 4). Notably, despite deregulation of cyclin D species in NT2/D1-R1 cells, repression of each D-type cyclin resulted in decreased proliferation (Fig. 4B). These findings indicate that these EC cells are still sensitive to growth repression by targeting D-type cyclins, despite RA resistance.

Differential expression of D-type cyclins in GCTs. Immunohistochemical analyses of clinical GCTs were performed in cases exhibiting both EC and teratoma components. Findings revealed that EC components of GCTs had a higher proliferative component than the adjacent teratoma (Fig. 5). A representative case is shown in Fig. 5. Cyclin D1 immunostaining revealed that highly proliferative regions of GCTs had higher nuclear and cytoplasmic cyclin D1 than the adjacent teratoma where staining was restricted to the nucleus. In contrast, cyclin D3 expression was less readily detected than cyclin D1 in the same cells and was preferentially expressed within teratomatous elements not exhibiting Ki-67 expression. A total of 36 samples were analyzed that contained EC and adjacent teratoma and 34/36 exhibited this pattern of expression. Thus, an inverse relationship was found to exist between cyclin D1 and cyclin D3 expression during *in vitro* and *in vivo* GCT differentiation.

Erlotinib treatment of RA sensitive and resistant EC cells (Fig. 6). Erlotinib treatment resulted in growth inhibition and repression of cyclin D1 levels in both NT2/D1 and the RA resistant NT2/ D1-R1 cell line, which is coresistant to cisplatin

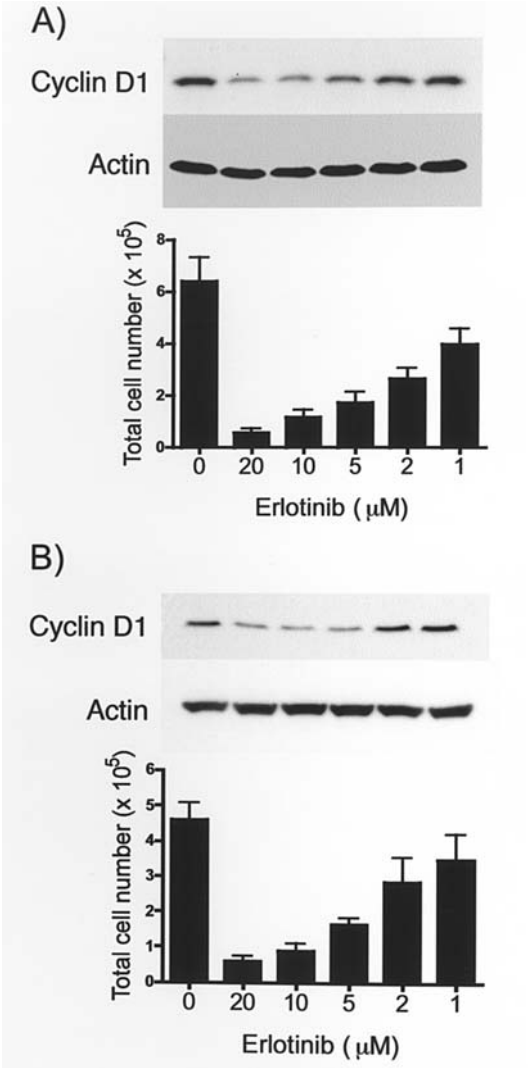

Figure 6. Erlotinib represses cyclin D1 levels and inhibits cell growth in (A) RA sensitive and (B) resistant NT2/D1 cells. Western blot analysis of cyclin D1 after 24 h Erlotinib treatment. Cell counts after 72 h Erlotinib treatment. Drug administered at 0 and 48 h.

(38). Cyclin D2 and cyclin D3 levels were not significantly changed (data not shown). Erlotinib levels of around 1-2 μM are clinically achievable and this dose is growth inhibitory for both cell lines. Specifically targeting cyclin D1 bypasses the RA and cisplatin resistance mechanism in NT2/D1-R1 cells causing growth suppression. Erlotinib may have clinical potential in patients who have recurrent disease after standard cisplatin-containing regimens.

Discussion

Transcriptional regulation of cyclin D1 has been reported as an outcome of multiple mitogenic signaling pathways. Over 50 regulators are cited as acting through the cyclin D1 promoter, as reviewed (36). It was therefore hypothesized that cyclin D1 mRNA repression following RA-treatment was most likely due to a decrease in transcription and not a change in message stability. The hnPCR study verified this as less unspliced cyclin D1 mRNA was being made after 72-96 h of RA-treatment. Initial transient transfection assays using luciferase-cyclin D1 promoter constructs did not establish RA-mediated repression of cyclin D1 promoter activity. However, when this construct was stably integrated into the NT2/D1 genome, RA-mediated repression was evident. Retinoids have been reported to repress cyclin D1 expression in multiple systems including human hepatoma cells, squamous cell carcinoma cells, neuroblastoma cells and breast cancer cells (39-43). In some cases, transcriptional repression has been found, however, the precise mechanisms engaged could differ between systems. For example, it was reported that retinoids suppressed insulin-dependent cell growth and cyclin D1 gene expression in human breast cancer cells (43), but that pathway may not be active in all cell contexts.

In clinical GCTs studied here, cyclin D1 expression was associated with a high proliferative state such as in EC elements, whereas cyclin D3 was seen predominantly in cells which were less proliferative as determined by Ki-67 immunostaining, such as teratomatous elements of the same GCT. In normal mouse testis cyclin D levels were examined and cyclin D1 was expressed only in proliferating gonocytes and spermatogonia whereas cyclin D3 was seen in both proliferating and quiescent gonocytes during testis development (44). Cyclin D3 expression was also seen in terminally differentiated Sertoli cells, in Leydig cells, and in spermatogonia in adult testis. Therefore, in spermatogonia, cyclin D1 and cyclin D3 seem to be involved in cell cycle regulation, however, cyclin D3 likely plays a distinct role in Sertoli and Leydig cells. Similarly, in a study of D-type cyclins in adult human testis and testicular cancer, elevated cyclin D3 levels in the differentiated Leydig cells, in teratoma and in NT2/D1 cells induced to differentiate with RA were detected (23). D-type cyclins were not detected in proliferating germ cells and in contrast to the current study, cyclin D1 was undetected in NT2/D1 cells. In the current analysis of cyclin D1 expression in clinical GCTs, it is clearly elevated in EC compared to teratoma and was readily detected in NT2/D1 cells. Any discrepancy between these two studies is likely due to the different antibodies used for cyclin D1 immunodetection. In prior work, cyclin D2 levels were elevated in most carcinoma *in situ* GCT lesions studied, implicating a role for this D-type cyclin in early germ cell tumorigenesis (23). Levels of cyclin D2 were not significantly different between EC and teratoma (23) in agreement with the NT2/D1 data, which is why cyclin D2 was not analyzed in our series of clinical samples.

Induction of cyclin D3 levels with differentiation has been reported in other systems including terminal differentiation of skeletal myoblasts (24). In this study of NT2/D1 cells, elevation of cyclin D3 was seen only at the protein level whereas the repression of cyclin D1, although initially due to accelerated proteolysis (12), was followed by transcriptional repression of cyclin D1 as shown in Fig. 1. Several groups have suggested that increases in cyclin D3 levels result from interactions with the cell cycle inhibitor p27 (31,45). In p27 null murine embryonic stem (ES) cells induced to differentiate with RA, cyclin D3 expression is much lower when compared to normal ES cells and these cells also have a higher rate of apoptosis (46). Together, these findings suggest a role distinct from the phosphorylation of Rb for the high levels of cyclin D3 expression found in GCTs.

Specific siRNA-mediated repression of each cyclin D species causes growth repression, however, it is only cyclin D1 that is repressed when EC cells undergo terminal differentiation following RA-treatment or when clinical GCTs undergo maturation from EC to mature teratoma. Prior studies have sought to determine whether the three cyclin D species have distinct roles in normal growth, development and tumorigenesis. Transgenic mice engineered to over-express different D-type cyclins in the breast, under the control of the mouse mammary tumor viral promoter, produced three distinct phenotypes. MMTV-cyclin D1 mice produced breast adenocarcinoma and squamous cell carcinoma (SCC). MMTV-cyclin D2 mice show a lack of alveologenesis during pregnancy and only develop carcinoma at low frequency and MMTV-cyclin D3 mice only develop SCC (47). Yet, in studies culminating in the knockout of all three cyclin D species, murine D-cyclins were dispensable for proliferation of numerous cell types (26,48). However, these same species are not dispensable for transformation by RAS- and MYC-driven oncogenic pathways and therefore they represent attractive molecular targets for cancer chemoprevention or therapy (26). Cyclin D1 only knockout mice are resistant to breast cancers induced by Ras and ErbB2 oncogenes, but not by Wnt or c-Myc (49). Cyclin D1 deficient mice are also resistant to gastrointestinal tumors arising in the ApcMin mice (50). Lack of D-type cyclins or Cdk4 or Cdk6 results in cells with an impaired ability to enter the cell cycle from a quiescent state and to respond to activation of mitogenic signaling pathways, as reviewed (51). Cdk-4-null mouse embryonic fibroblasts (MEFs) senesce rapidly in culture (52). The combination of an enhanced ability to enter the cell cycle, response to mitogenic signaling and avoidance of senescence can account for the frequent deregulation of this pathway in cancer.

In conclusion, of the three D-type cyclins, cyclin D1 is preferentially repressed as GCTs are induced to differentiate both *in vitro* and *in vivo*. Also an inverse relationship was found to exist between cyclin D1 and cyclin D3 during this differentiation program. Agents that target cyclin D1 were hypothesized to repress the growth of GCT cells. Different siRNAs that target individual D-type cyclins were used to confirm this. To test this independently, EC cells were treated with the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. Growth inhibition was triggered in both NT2/D1 and NT2/D1-R1 cells and this also decreased cyclin D1 protein levels. Thus, cyclin D1 is a molecular target for the treatment of GCTs and agents that target this species should be explored, especially in cases that are resistant to cisplatin-based chemotherapy regimens.

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