Cyclin D1 Determines Mitochondrial Function In Vivo†

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The cyclin D1 gene encodes a regulatory subunit of the holoenzyme that phosphorylates and inactivates the pRb tumor suppressor to promote nuclear DNA synthesis. cyclin D1 is overexpressed in human breast cancers and is sufficient for the development of murine mammary tumors. Herein, cyclin D1 is shown to perform a novel function, inhibiting mitochondrial function and size. Mitochondrial activity was enhanced by genetic deletion or antisense or small interfering RNA to cyclin D1. Global gene expression profiling and functional analysis of mammary epithelial cell-targeted cyclin D1 antisense transgenics demonstrated that cyclin D1 inhibits mitochondrial activity and aerobic glycolysis in vivo. Reciprocal regulation of these genes was observed in cyclin D1-induced mammary tumors. Cyclin D1 thus integrates nuclear DNA synthesis and mitochondrial function.

The induction of tumorigenesis is a multistep process (23). Oncogenic and growth factor signals induce premature senescence in primary cells (6, 13, 28, 34). Local environmental cues regulate early events of tumorigenesis. Mouse embryo fibroblasts (MEFs) grown under reduced-oxygen conditions delay senescence and show less oxidative DNA damage (43). Premature senescence induced by oncogenic signals such as Ras or ErbB2 must be sequentially bypassed for cellular transformation to occur. The subsequent deregulation of growth control recruits altered genetic signals that sustain constitutive mitogen signals, deregulated cell cycle control, and altered cellular metabolism including changes in glycolysis (61). Like oncogenic stimuli, inactivation of glycolytic enzymes may trigger premature senescence (31). Conversely, glycolytic enzymes protect MEFs from both oncogenic reactive oxygen species production and senescence induction (31), demonstrating the importance of cellular metabolism in the early events of tumor initiation.

Mitochondria are key integrators of diverse metabolic signals. Mitochondria produce ATP through the coupling of electron transport with proton pumping (22). Metabolic activities of mitochondria include heme synthesis, single carbon metabolism, fatty acid metabolism, oxidative glycolysis, and production of reactive oxygen species. Aging and tumorigenesis are associated with mitochondrial DNA mutations, and mitochondrial function is being considered as a potential target for cancer therapies (12). The nuclear signals regulating mitochondrial function in vivo are poorly understood. Furthermore, the mechanisms regulating mitochondrial function during the onset and progression of tumorigenesis are largely unknown. Global gene expression profiling has proven powerful in capturing comprehensive molecular phenotypes reflecting biological mechanisms. Distinct subpopulations of gene expression have been identified within histologically similar tumors, with prognostic relevance likely reflecting distinct oncogenic driver events (20, 45, 63). Gene expression models in turn identified distinct gene clusters recruited by either the Ras or Myc oncogenes (20, 45, 63). By providing tight temporal and spatial control, inducible transgenics have facilitated the dissection of coincident from causal gene expression in tumors and identified early events regulated by Ras and c-Myc. The dissection of molecular genetic events regulated by oncogenic signals in vivo has provided important mechanistic insights, and molecular genetic signatures may prove useful in therapeutic stratification, prognostication, and early detection (20, 27).

The cyclin D1 gene, which encodes a regulatory subunit of the holoenzyme that phosphorylates and inactivates the retinoblastoma protein (pRb), is overexpressed in a variety of tumors, including breast cancer, often at the very early stage of ductal carcinoma in situ. cyclin D1 is a collaborative oncogene, and mammary-targeted cyclin D1 overexpression is sufficient for the induction of mammary adenocarcinoma in transgenic...
mice (60). Typically, cyclin D1-overexpressing human tumors have low proliferative indices (42, 52), and hierarchical clustering demonstrated that cyclin D1 expression is associated with the luminal epithelial phenotype (20, 45, 63). In contrast, tumors with cyclin E overexpression or pRb inactivation showed increased cellular proliferative indices, correlating with distinct gene clusters. Cyclin D1−/− mice are resistant to mammary tumors induced by oncogenic ErbB2 or Ras (65) but not Myc, suggesting cyclin D1 regulates oncoprotein-specific functions. In addition to the well-defined role in phosphorylation of the pRb and cell cycle control, cyclin D1 conveys cyclin-dependent kinase (CDK)-independent functions (18, 33, 59). Cyclin D1 regulates the transcriptional activity of C/EBPβ and PPARγ (59), both part of a common signaling pathway required for normal mammary gland development and adipogenesis (2).

Given the importance of cyclin D1 in tumorigenesis induced by diverse oncopgenic signals, the molecular genetic targets of cyclin D1 have been investigated. Analysis of cyclin D1−/− mice has revealed a requirement for cyclin D1 in diverse cell types, including mammary gland development (15, 53, 54) and normal function of blood vessels, macrophages, adipocytes, and hepatocytes (1, 25, 41, 59). Because mammary gland development and tumorigenesis involve heterotypic signals from each of these cell types, an analysis of cyclin D1 function in mammary epithelial cells in vivo requires the development of transgenic mice that temporally and spatially control cyclin D1 expression. Understanding the mechanism by which cyclin D1 governs cellular transformation requires the identification of genes regulated by cyclin D1 in the presence of oncogenic signals in vivo.

The transgenic mouse has been used extensively in the molecular analysis of genetic function. Homozygous deletion of genes that are ubiquitously expressed or conduct a critical function in normal cells may result in embryonic lethality, developmental abnormality, or compensation by alternate genes within the same cell. Transgenic mouse models that convey spatial and temporal control have been used to more effectively recapitulate human disease and analyze gene function in vivo, using ligands including tetracycline, steroid hormones (RU486 or Tamoxifen), or chemical inducers of dimerization (2). The edcysone system has the advantages of low basal-level expression and high inducibility in cultured cells; however, transgenic analyses had previously been limited by the lack of availability of sufficient highly bioactive edcsyroides for in vivo analysis (49).

To understand genetic targets regulated by cyclin D1 in oncogenic signaling, the edcysone system was developed herein to regulate cyclin D1 antisense expression in the mammary epithelia of transgenic mice expressing ErbB2. Mammary epithelial cyclin D1 antisense induced genes governing mitochondrial function and glycolysis. Reciprocal expression of these genes was observed in mammary tumors induced by mammary gland-targeted cyclin D1 overexpression. Reduction in cyclin D1 abundance by antisense knockdown in transgenic mammary epithelia or by small interfering RNA (siRNA) in normal or transformed breast cancer cells recapitulated the selective changes in mitochondrial activity and glucose metabolism. In addition to regulating nuclear DNA synthesis, cyclin D1 regulates mitochondrial function in vivo, coordinating metabolic substrate utilization within the cell. 

MATERIALS AND METHODS
Ponasterone-inducible cyclin D1 antisense-IRES-GFP transgenic mice. Exper- imentation was approved by the Georgetown University and Albert Einstein College of Medicine animal use committees. Genotyping for each of the five transgenes co-integrated in the ErbB2-cyclin D1 antisense lines was performed by either genomic Southern analysis (3, 35) or by PCR of genomic DNA. The mouse mammary tumor virus (MMTV)-VgErCdr/RXRα and EGRE/βgal (3), cyclin D1 antisense internal ribosome entry site (IRES)-green fluorescent protein (GFP) (59), and MMTV-ErbB2 transgenic mice were previously described (35). Virgin mice 70 days of age at a similar phase of the menstrual cycle were used. Ponasterone A (200 μg) pellets (Innovative Research, Sarasota, Florida) (3) were implanted into the interscapular region of mice at 50 to 55 days of age for a total of 18 to 21 days. β-Galactosidase staining was performed as previously described (3).

RNA isolation, oligonucleotide microarray, multidimensional scaling, and cluster analysis. Total RNA was isolated from age-matched mouse abdominal mammary glands or MMTV-cyclin D1 mammary tumors as described previously (26) and used to probe Affymetrix U74Av2 arrays (Affymetrix, Santa Clara, California). Data generated after scanning were normalized and subjected to comparison analysis to select “change calls”. Comparisons were made between the placebo- and ponasterone A-treated ErbB2-cyclin D1 antisense transgenic mice, and a change call list was generated. Additional comparisons were made between the placebo- and ponasterone A-treated ErbB2-control transgenic mice to identify genes affected by ponasterone A. In another experiment, comparisons were made between the placebo- and ponasterone A-treated ErbB2 transgenic mice and MMTV-cyclin D1 transgenic mice, and a change call list was generated. In two sets of three arrays, nine change calls were generated, with at least eight consistently increased or decreased. The data selected after compar- ison analysis were further filtered based on absolute analysis using the Mann-Whitney U test, and detection calls and genes with significant differences in expression were selected for multidimensional scaling and hierarchical cluster- ing. Multidimensional-scaling coordinates were calculated with Matlab software. Distances between samples were calculated using the Pearson correlation coeffi- cient (distm = 1 − Pearson correlation coefficient). To visualize expression of the selected genes, intra- and intersample pairs hierarchical clustering was per- formed using Cluster 3.0 (Stanford University). A gene list corresponding to clusters was generated using the Data Mining Tool from Affymetrix.

Cell culture, retroviral infection, siRNA transfection, and reporter assays. 293T, MCF7, MCF10A, and NAFa mammary cell lines were cultured (3, 35) and the culturing of primary murine mammary epithelium from the transgenic mice was conducted as described previously (32). The cdk4+/−: 3T3 fibroblasts were previously described (48), cyclin D1−/−: 3T3 cells were derived from cyclin D1−/−: MEF (4). The retroviral expression vector for cyclin D1 antisense was constructed using the fragment of cyclin D1 antisense-IRES-GFP subcloned as an EcoRI fragment into the retroviral vector mouse stem cell virus LTR and viral enhancer (43). Total RNA to cyclin D1 (5′-CAGCUCAAGUGGAACCUG-3’, 5′-CAGGULLCACUUGAGCUUG-3’) and control nonsilencing siRNA were obtained from QIAGEN (Valencia, California). The transfection of siRNA duplexes was performed with the manufacturer’s protocol for Oligofectamine reagent (Invitrogen, Carlsbad, California). The hexo- kinase II promoter-luciferase reporter construct (−4396-HKII-Luc) was kindly provided by Peter L. Pedersen (The Johns Hopkins University, Baltimore, Maryland). The expression vector for mouse cyclin D1 was constructed using the fragment of pGEM7-mouse cyclin D1 subcloned as an EcoRI fragment into pcDNA3. The −4396-HKII-Luc plasmid (1 μg) was transfected using GeneJuice reagent (Novagen, Madison, Wisconsin) into NAFa cells, together with either empty or cyclin D1 expression vector. At 36 h posttransfection, a luciferase assay was conducted as previously described (59). The relative firefly luciferase activ- ities were calculated by normalizing transfection efficiencies according to either Renilla luciferase or β-galactosidase activities, which gave identical trends.

Western blotting and immunohistochemistry. Western blot analysis was con- ducted as previously described (41). Total cellular lysates (50 μg) prepared from subconfluent cultures were separated by 8% sodium dodecy1 sulfate-polyacryl- amide gel electrophoresis and transferred electrochemically to a polyvinylidene fluoride membrane. After being blocked with 5% dry milk in phosphate-buffered saline (PBS), the membranes were probed with the specific primary antibodies described below. The appropriate horseradish peroxidase-conjugated secondary antibodies were subsequently applied, and immunodetection was achieved using the enhanced chemiluminescence procedure.

Immunohistochemistry for detection of cyclin D1 was carried out as per the protocol (http://www.chemicon.com/techsupp/protocol/paraffinprotocol.asp) available from Chemicon International. Primary antibody (AB-3) was used at a
1:50 dilution, and secondary goat anti-rabbit antibody–horseradish peroxidase was used at 1:250. The Dako liquid DAB staining system (Dako Corporation, Carpinteria, California) was used to visualize target antigen. Selected sections were counterstained with hematoxylin.

**Antibodies.** The following antibodies were used in these experiments: Ab-3, rabbit polyclonal antibodies to cyclin D1 (Lab Vision/Neomarker, Fremont, California); 1:21, mouse monoclonal antibodies (MAb) to VP16; H-98, rabbit polyclonal antibodies to insulin-like growth factor binding protein-3; 9E10, mouse MAb to fatty acid synthase (BD Biosciences, San Diego, California); 07-439, rabbit polyclonal antibodies to NADH:ubiquinone oxidoreductase (complex I) subunit 1 (Santa Cruz Biotechnology, Santa Cruz, California); 23, mouse MAb to fatty acid synthase (BD Biosciences, San Diego, California); Ab-1, rabbit polyclonal antibodies to c-Neu (Oncogene Research Products, San Diego, California); 1-21, mouse monoclonal antibodies (MAb) to VP16; H-98, rabbit polyclonal antibodies to insulin-like growth factor binding protein-3; 9E10, mouse MAb to fatty acid synthase (BD Biosciences, San Diego, California); 07-439, rabbit polyclonal antibodies to NADH:ubiquinone oxidoreductase (complex I) subunit 1 (Santa Cruz Biotechnology, Santa Cruz, California).

**Northern blotting.** Northern blot analysis was conducted as previously described (9). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California). The riboprobes were generated by in vitro transcription of pGEM7-S (Santa Cruz Biotechnology, Santa Cruz, California).

**TABLE 1. RT-PCR primers used**

| Gene product | Application | Orientation | Sequence | Annealing temp (°C) |
|--------------|-------------|-------------|----------|-------------------|
| ErbB2        | Genomic PCR | Forward      | 5'-CGGAACCCACATCACGGCC-3' |
|              |             | Reverse      | 5'-TTTCCTCAGCCTACGGC-3' |
| VgEcR        | Genomic PCR | Forward      | 5'-TTTCGACGCACTGGATGAA-3' |
|              |             | Reverse      | 5'-GGTTGTAATACCTCAGG-3' |
| RXRα         | Genomic PCR | Forward      | 5'-TGGCAGTACATCAAGTGATACA-3' |
|              |             | Reverse      | 5'-GAGTGTAATGACGAGGAGA-3' |
| Cyclin D1    | Real-time RT-PCR | Forward | 5'-GCAAGCCATGACAGACCTT-3' |
|              |             | Reverse      | 5'-GTTTGGCCGGATGACAGG-3' |
| Cyclin D1    | Genomic PCR and RT-PCR | Forward | 5'-GCCACCTGGATGCTGGAGG-3' |
|              |             | Reverse      | 5'-AACCTTGTACCTGATGAA-3' |
|              | Real-time RT-PCR | Forward | 5'-TCTCTGAGTACGAGGTAT-3' |
|              |             | Reverse      | 5'-GTTTCCCAGTACGACG-3' |
| β-Gal        | Genomic PCR | Forward      | 5'-CCCCCCGCGATATCCC-3' |
|              |             | Reverse      | 5'-GTTTTTCACAGTCAGGAC-3' |
| RPL-19       | Real-time RT-PCR | Forward | 5'-AATGCGTCCGATGCTGAGA-3' |
|              |             | Reverse      | 5'-CTCCATGAGATGGGCTTG-3' |

**TABLE 2. Probes used for FISH**

| Target Name | Gene family | Application | Orientation | Sequence |
|-------------|-------------|-------------|-------------|----------|
| Sens cyclin D1 detection | D1.1 | 5'-GAGATTTGGTCACCTGCGGGAAATCGTGGCCACCTGGATGCTGGAGG-3' |
| Sens cyclin D1 detection | D1.2 | 5'-GGATTTGGTCACCTGCGGGAAATCGTGGCCACCTGGATGCTGGAGG-3' |
| Sens cyclin D1 detection | D1.3 | 5'-TCCATTTGACAGCCTCCTCCGTCGGGCGGATAGTTGCTGTAGTGTAGTGC-3' |
| Sens cyclin D1 detection | D1.4 | 5'-GATTACACCTGAGGCTGGTTGGGAAATGAACTTACATCCTGCGGCACAG-3' |
| Sens cyclin D1 detection | D1.5 | 5'-TCTCTAGAACCAGAAGCAACATTTAAATAGAACTAATAGTAAATT-3' |
node. The numbers of branches represent the means of branching numbers along the three longest ducts.

**FISH.** Fluorescence in situ hybridization (FISH) was performed as described, with modifications (14). The tissue slides were deparaffinized with xylene (Fisher, Missouri), dehydrated in 100% ethanol followed by 95% ethanol, and rinsed with distilled water followed by a PBS (pH 7.0) wash. Citrate buffer (9 ml 0.1 M citric acid monohydrate, 41 ml 0.1 M sodium citrate, and 450 ml distilled water, pH 6) was prewarmed in a pressure cooker for 5 min. Tissue slides were added to the prewarmed citrate buffer and microwaved in the pressure cooker for 8 min. After cooling, the tissue slides were rinsed several times with distilled water and added to a 0.5% sodium borohydride solution (in 1× PBS) for 30 min. Last, the tissue slides were washed several times with distilled water, rinsed with PBSTM (1× PBS-5 mM MgCl₂), and equilibrated in pre-/post-hybridization wash (50% formamide and 50% 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) for 10 min. Twenty nanograms of each of the following probes was aliquotted into one Eppendorf tube per tissue sample: cyclin D1 (D1.1 to -5) in Cy3, antisense cyclin D1 (TG1 to -5) in Cy5, and antisense cyclin D1 (TG1 to -5) in Cy5. Competitor (50 µl 10 mg/ml sheared salmon sperm DNA and 50 µl 10 mg/ml Escherichia coli rRNA) was added in 100-fold excess (with respect to total probe concentration) to each tube and vacuum dried. The dried pellets were resuspended in 10 µl of formamide on a heating block at 85°C for 5 to 10 min and then immediately placed on ice. Ten microliters of hybridization buffer (20 µl 20× SSC, 20 µl bovine serum albumin, and 60 µl distilled water) was added to each tube. A glass plate was wrapped with paraffin, and each tissue slide was dried and placed on the paraffin face up. A 20× reaction was dotted onto each glass slide, and coverslips were placed on each slide. The slides were covered and sealed with nail polish, incubated for 3 hours at 45°C, and then washed in a 5× SSC wash at 37°C. After incubation, the top layer of paraffin was carefully removed, and the slides were placed in pre-/post-hybridization wash for 5 min to allow the coverslips to come off. This wash was repeated twice more for 20 minutes with the coverslips off. The tissue samples were then washed with 20× SSC for 10 minutes and PBSTM for 10 minutes. The slides were then stained with DAPI (4′,6-diamidino-2-phenylindole; for staining, 100 ml 10× PBS stock plus 50 µl 10 mg/ml DAPI stock was used) for 1 minute and rinsed with PBSTM. Each glass slide was mounted with a coverslip using freshly prepared AntiFade mounting medium (Molecular Probes, Oregon).

**FISH probe targets, names, and respective sequences are shown in Table 1.**

**Microarray probe synthesis and hybridization.** Total RNA was amplified according to the Eberwine procedure using a MessageAmp kit (Ambion, Austin, Texas). During in vitro transcription, biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, New York) were incorporated. Twenty micrograms of the biotinylated cRNA product was fragmented at 94°C for 35 min before being used for hybridization. Hybridization to a set of two Affymetrix MG-U74Av2 GeneChips was performed overnight, followed by staining and washing as per the manufacturer’s instructions. The processed chips were then scanned using an Agilent GeneArray scanner. Grid alignment and raw data generation were performed using Agilent GeneChip 5.0 software. For quality control, oligonucleotide B2 was hybridized to analyze the checkerboard pattern in each corner of the chip, and bioB, bioC, and bioD probes were added to each sample with various concentrations to standardize hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization intensity between oligonucleotides that perfectly matched the transcript sequence and oligonucleotides containing single-base-pair mismatches, were calculated. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures. Raw expression values, representing the average difference in hybridization, staining, and washing procedures.
FIG. 2. Inducible mammary epithelial cell-targeted cyclin D1 antisense transgene expression in vivo. (A) Schematic representation of ponasterone-inducible cyclin D1 antisense-ErbB2 transgenes (right panel) and “ErbB2-control line” (left panel). CMV, cytomegalovirus. (B) PCR analysis and (C) genomic Southern blot of transgenes integrated into transgenic mice lines. (D) Mammary gland squashes of separate transgenic mice treated with either placebo or ponasterone A pellets. (E) Quantification of mammary gland branch numbers is shown for n = 3 separate transgenic mice in each group. (F) β-Galactosidase expression in mammary glands of cyclin D1 antisense lines treated with either placebo or ponasterone A pellets. (G) RT-PCR analysis of mammary epithelium from control or cyclin D1 antisense mice treated with ponasterone A. (H) FISH of mammary epithelium from cyclin D1 antisense-ErbB2 transgenic mice treated with ponasterone A. Single cells are shown with arrows indicating the presence of cyclin D1 antisense transgenic transcript. b-gal, β-galactosidase; A/S, antisense.
copy. Once a gland was located, a "voxel" 0.15 cm on edge (3.375/9262
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was acquired with a spectral width of 4 kilohertz and processed using a line broadening of 4 Hz. Shorter spectra were also acquired prior to this, to rule out contribution to the 1.3 ppm resonances resulting from anesthesia (36, 37, 55). Glutamate + glutamine peaks are integrated and assigned as "Gx" in the 1.8- to 2.6-ppm range.

RESULTS

Effect of cyclin D1 on hexokinase expression and function. Global gene expression profiling previously identified gene clusters that separated ErbB2 versus Myc-induced mammary tumors (8, 26). Myc induction correlated with expression of proliferation-related genes and the induction of mitochondrial gene expression. Myc is known to induce the expression of genes promoting aerobic glycolysis, including hexokinase II (30). Western blotting analysis of mammary tumors derived from transgenic mice in which the MMTV promoter was used to drive either c-Myc or ErbB2 demonstrated increased abundance of hexokinase II in tumors induced by c-Myc compared with ErbB2. Similar levels of total protein abundance were shown using the internal control GDI. Cyclin D1 levels were relatively higher in ErbB2- than c-Myc-induced tumors (Fig. 1A). Consistent with previous studies demonstrating the induction of cyclin D1 by ErbB2 (35), MCF10A human breast immortalized epithelial cells transformed with retroviral expression vectors encoding either an activating ErbB2 mutant, both Ras and ErbB2, or c-Myc demonstrated a greater induction of hexokinase II in MCF10A cells transformed with c-Myc compared with either ErbB2 or Ras and ErbB2. Western blotting showed similar levels of protein abundance and the presence of either the ErbB2 protein in ErbB2-transformed cells or c-Myc in c-Myc-transformed MCF10A. Cyclin D1 levels were increased in both the NeuT- and Ras/ErbB2-transformed MCF10A compared with the c-Myc-transformed cells (Fig. 1B).

As cyclin D1 levels were increased in mammary tumor cells with reduced hexokinase II abundance, we examined the possibility that cyclin D1 may contribute to the altered expression of hexokinase II in the immortal cell line MCF10A. NeuT-MCF10A cells were therefore transduced with a retrovirus expressing cyclin D1 linked to an IRES-GFP, and GFP sorting was conducted. Cyclin D1 protein induction reduced hexokinase II abundance (Fig. 1B, lane 6 versus lane 7). ErbB2 levels were unchanged. The hexokinase II enzymatic activity of the oncprotein-transduced MCF10A cells correlated with hexokinase II protein abundance. Transduction of MCF10A-NeuT cells with cyclin D1 reduced hexokinase II activity (hexokinase II activity NeuT/GFP, 37.5 ± 0.6 versus NeuT/cyclin D1-IRES GFP, 20.9 ± 0.49; P < 0.05) (Fig. 1C). Transcriptional activities of the hexokinase II promoter, which is induced by growth factors, Myc, and mutant p53, were inhibited in a dose-dependent manner by coexpression of cyclin D1 in cells derived from MMTV-ErbB2 transgenic mice (NAFA). Furthermore, cyclin D1 antisense induced hexokinase II promoter activity (P < 0.001) (Fig. 1D). To determine whether the effect of cyclin D1 on hexokinase II promoter activity was cyclin specific, comparisons were made with cyclin D3, cyclin A, and cyclin E in NAFA cells. Cyclin D3 and cyclin E did not repress the hexokinase II promoter (Fig. 1E). Cyclin A expression induced the hexokinase II promoter, in contrast with cyclin D1. Collectively, the studies demonstrate that the repression of hexokinase II by cyclin D1 is cyclin specific. Hexokinase II promoter activity was fourfold more active in cyclin D1 cells than cyclin D1 cells, and expression of cyclin D1 inhibited hexokinase II promoter activity in cyclin D1 cells (Fig. 1F). Together these studies demonstrate that cyclin D1 inhibits the expression and activity of hexokinase II in breast cancer cells. Growth factor addition, c-Myc expression, and cyclin D1 expression are each capable of inducing mammary epithelial cell DNA synthesis, but growth factors and cyclin D1 expression have opposing effects on hexokinase II abundance and transcription. Thus, the effect of cyclin D1 on hexokinase II is not an indirect effect on DNA synthesis, which would be expected to increase hexokinase II activity.

Sustained in vivo regulation of mammary epithelial cell-targeted cyclin D1 antisense in transgenic mice. As cyclin D1 inhibited hexokinase II expression and activity induced by ErbB2 in cultured breast cancer cells, transgenic mice were generated to assess the role of cyclin D1 in regulating this function in vivo. As cyclin D1-deficient mice fail to develop normal terminal alveolar breast buds and demonstrate abnormalities of macrophage and blood vessel function (1, 25, 41, 59), inducible cyclin D1 antisense mice were generated to allow the regulation of cyclin D1 abundance selectively in the normally developed mammary gland within the mammary epithelial cell. Transgenic mice were generated in which the cyclin D1 antisense IRES-GFP, driven by the ecdysone enhancer, was targeted to the mammary epithelium using the MMTV-driven VgEcR (3) (see Fig. S1 and S2 in the supplemental material). These mice were mated to the MMTV-ErbB2 transgenic mice, and the resulting lines cointegrate five transgenes (Fig. 2A). Comparison was made between the onpanasterone-inducible cyclin D1 antisense lines and the control lines, which express four transgenes including the β-galactosidase reporter gene. Transgene transmission was monitored by genomic Southern blotting and PCR (Fig. 2B and C). The morphology of the mammary gland was determined by mammary squash, and the number of ductal branches was assessed (21). The addition of onpanasterone A did not affect either the number of branches or terminal end buds in ErbB2-control or ErbB2-cyclin D1 antisense transgenic mice (Fig. 2D and E). β-Galactosidase reporter gene expression was observed in the mammary epithelia of mice implanted with onpanasterone A pellets (Fig. 2F). The cyclin D1 antisense transcript was readily detectable by RT-PCR of the transgenic mammary epithelium of ErbB2-cyclin D1 antisense lines but not in the ErbB2-control line animals (Fig. 2G). To demonstrate reduced cyclin D1 abundance in onpanasterone A-treated ErbB2-cyclin D1 antisense transgenic mice, immunohistochemistry against cyclin D1 was conducted on serial sections from placebo and onpanasterone A-treated mice. Immunoperoxidase staining for cyclin D1 protein demonstrated a reduction in cyclin D1 abundance in mammary epithelial cells of cyclin D1 antisense mice treated with onpanasterone A (see Fig. S3 in the supplemental material). To identify, at a single-cell level, the expression of cyclin D1 antisense expression in transgenic mammary epithelium, FISH analysis was conducted. Probes directed to the cyclin D1 antisense transcript detected the induction of cyclin D1 antisense in the mammary epithelium of transgenic mice but not control
FIG. 3. Cyclin D1 antisense induces hexokinase II and genes governing oxidative glycolysis and mitochondrial function in vivo. (A) Treeview display of microarray expression data comparing mammary epithelium of ponasterone A- or placebo pellet-treated cyclin D1 antisense/ErbB2 transgenics. Levels of expression are shown for upregulated genes (red) and downregulated genes (green). Raw gene expression data and gene names and accession numbers are shown at http://www.jci.tju.edu/pestell/papers/CD1AS (worksheet labeled “252 genes”). (B) Pearson correlation coefficient analysis of ponasterone A- or placebo pellet-treated ErbB2-cyclin D1 antisense transgenics. Separation of gene groups by metagene
mice or antisense mice in the absence of ponasterone A (Fig. 2H and data not shown).

Cyclin D1 antisense induces mitochondrial and lipogenic regulatory gene clusters in vivo. Gene expression profiling of human breast cancer samples using factor models and Bayesian regression methodologies suggests that expression of groups of genes may define tumor estrogen receptor status or basal versus luminal cell type of tumors (63). Thus, statistical approaches can be used to discriminate samples based on properties reflecting the underlying biology. mRNA from the cyclin D1 antisense transgenic mice was therefore subjected to microarray analysis. Comparison was made between mice treated with ponasterone A and placebo. Profiles of gene expression were normalized and 252 genes (263 probe sets) were selected by the procedure outlined in Materials and Methods. Individual gene expression profiles were generated using Treeview (Fig. 3A). A relative decrease in expression is represented by green and increased expression is represented by red. To determine functional relationships among selected genes, we used the NetAffx Gene Ontology Mining Tool and extracted keyword annotations in an automated manner (Table 3). Genes that were reproducibly differentially expressed in the mammary epithelia of at least three independent cyclin D1 antisense transgenic mice are shown (Fig. 3A). Of the 252 genes, 3 genes were regulated by ponasterone A treatment alone (Fig. 3A; please see http://www.jci.tju.edu/pestell/papers /CD1AS/ for individual gene names). To determine whether global patterns of gene expression are altered by cyclin D1 antisense, pairwise distances between ponasterone A-treated and untreated animals were calculated using the Pearson correlation coefficient and were visualized in three-dimensional space using multidimensional scaling (Fig. 3B). Similar methods had been used in the past to represent global relationships between tumors at the level of gene expression (8, 26) and to understand mammary gland development (40).

These analyses of mammary gland gene expression demonstrated that the cyclin D1 antisense mammary epithelium occupied discrete regions of gene expression space that was separable from other surveyed points (Fig. 3B). Microarray analysis demonstrated that cyclin D1 antisense induced the expression of genes that enhance oxidative glycolysis, lipogenesis, and mitochondrial function. To determine whether the alterations in gene expression identified by microarray analysis correlated with alterations in protein expression for these pathways in vivo, Western blot analyses were conducted of the mammary epithelium from the transgenic mice to assess the abundance of key regulators of glycolysis (hexokinase II, pyruvate kinase) and lipogenesis (fatty acid synthase, acetyl-CoA carboxylase). Two distinct founder lines expressing the cyclin D1 antisense transgene were examined to avoid any potential position of transgene integration effects. Ponasterone A treatment induced expression of the β-galactosidase reporter transgene (Fig. 3D). Normalization for protein loading was conducted using GDI. Consistent with the microarray analysis, the induction of cyclin D1 antisense in the mammary epithelial cell induced acetyl-CoA carboxylase, fatty acid synthase, hexokinase II, and pyruvate kinase (Fig. 3C and D). In view of the induction of mitochondrial genes reflecting mitochondrial function, we assessed the abundance of a representative protein encoded by mitochondrial DNA [NADH:ubiquinone oxidoreductase (complex I) subunit 1 (ND1)]. The abundance of ND1 was increased 4.6-fold in the mammary epithelium of cyclin D1 antisense mice (Fig. 3D). The same changes in protein abundance were observed in both cyclin D1 antisense founder lines, making the observed changes unlikely to be secondary to transgene site-of-integration effects. Similar observations were made in the cyclin D1<sup>−/−</sup> mammary epithelium compared with littermate controls (see Fig. 8A).

During murine development, cyclin D1 deficiency results in defective pregnancy-induced terminal alveolar breast bud development and in retinal apoptosis (15, 53). These abnormalities are rescued through expression of cyclin E, suggesting that these developmental abnormalities are consequences of reduced expression of either cyclin D1 or cyclin E. We therefore considered the possibility that cyclin E may also be reduced in cyclin D1 antisense mammary epithelium and was therefore unable to compensate for the reduction in cyclin D1 expression. The mRNA level for cyclin E was unchanged by reduced cyclin D1 abundance (data not shown). As cyclin E is readily detectable and unchanged in abundance in cyclin D1 antisense mammary gland, these studies suggest cyclin E does not compensate functionally for the reduction in cyclin D1 abundance.

Target genes in cyclin D1-overexpressing mammary tumors. Inspection of the function of genes regulated by cyclin D1 antisense demonstrated the induction of nuclear genes promoting oxidative glycolysis, lipogenesis, and mitochondrial function in mammary epithelium (Fig. 4). Previous studies have examined clusters of genes associated with cyclin D1 overexpression in tumors (33, 59). To determine which genes are regulated in tumors induced by cyclin D1 overexpression, microarray analysis was conducted of MMTV-cyclin D1-induced mammary gland tumors, compared with control mammary gland (placebo-treated transgenic cyclin D1 antisense mice) (Fig. 5A). A quantity of 1,925 genes were differentially regulated in the MMTV-cyclin D1 tumors. Such studies do not distinguish genes associated with tumorigenesis induced by cyclin D1 from those genes regulated by cyclin D1. To identify genes regulated by cyclin D1 that may contribute to mammary tumorigenesis, comparison was made between MMTV-cyclin D1-regulated genes and those genes regulated directly by the mammary gland-inducible cyclin D1 antisense. Expression profiling of mammary tumors from MMTV-cyclin D1 tumors revealed 31 genes that were reciprocally regulated compared with the genes regulated by cyclin D1 antisense in the mam-
TABLE 3. Genome-wide analysis of mRNA changes induced by the induction of cyclin D1 antisense in transgenic mice

| GenBank accession no. | Gene product name | Function or description* | Fold change* |
|-----------------------|-------------------|--------------------------|--------------|
| **Mitochondrion**     |                   |                          |              |
| AJ001418              | Pdk4              | Pyruvate dehydrogenase kinase, isoenzyme 4 * | −2.71 |
| L02914                | Aqp1              | Aquaporin 1 *            | −1.69 |
| AF041054              | Bnip3             | BCL2/adenovirus E1B 19-kDa interacting protein 1, NIP3 * | −1.68 |
| AW230209              | Mrps18a           | Mitochondrial ribosomal protein S18A * | 1.31 |
| AI846849              | Mrps18b           | Mitochondrial ribosomal protein S18B * | 1.43 |
| AW124133              | 3010027G13Rik     | RIKEN cDNA 3010027G13 gene | 1.58 |
| AA655369              | Timm8a            | Translocase of inner mitochondrial membrane 8 homolog a (yeast) [Saccharomyces cerevisiae] * | 1.59 |
| AW125336              | Pdhb              | Pyruvate dehydrogenase (lipoyamide) beta * ** | 1.66 |
| AW122428              | Timm10            | Translocase of inner mitochondrial membrane 10 homolog (yeast) | 1.94 |
| AA868883              | Scl25a10          | Solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10 * ** | 1.95 |
| AW1218217             | Scl25a1           | Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1 * ** | 2.01 |
| AI848354              |                      |                          |              |
| **Extracellular matrix** |                 |                          |              |
| M70642                | Ctgf              | Connective tissue growth factor * ** | −2.86 |
| AI844853              | Spock2            | SPARC/osteonectin, cwcv and kazal-like domains proteoglycan 2 | −1.83 |
| U26437                | Timp3             | Tissue inhibitor of metalloproteinase 3 | −1.74 |
| L19932                | Tgfbi             | Transforming growth factor, beta induced * | −1.67 |
| U06166                | Sparc1            | SPARC-like 1 (mast9, hevin) | −1.62 |
| AV375788              | Tdxb              | Tenascin XB *            | −1.57 |
| U43541                | Lamb2             | Laminin, beta 2 *       | −1.49 |
| X58861                | Clqa              | Complement component 1, q subcomponent, alpha polypeptide * ** | −1.49 |
| M22531                | ClqB              | Complement component 1, q subcomponent, beta polypeptide * | −1.34 |
| AV378405              | Entpd2            | Ectonucleoside triphosphate diphosphohydrolase 2 * | −1.31 |
| **Metabolism**        |                   |                          |              |
| AA869395              | Fohl1             | Folate hydrolase         | −21.57 |
| AJ001418              | Pdk4              | Pyruvate dehydrogenase kinase, isoenzyme 4 * | −2.71 |
| X78445                | Cyp1b1            | Cytochrome P450, 1b1, benz[a]anthracene inducible | −2.67 |
| AF009605              | Pck1              | Phosphoenolpyruvate carboxykinase 1, cytosolic | −2.61 |
| M74570                | Aldh1a1           | Aldehyde dehydrogenase family 1, subfamily A1 | −2.51 |
| U44389                | Hpgd              | Hydroxyprostaglandin dehydrogenase 15 (NAD) | −2.4 |
| L02331                | Sult1a1           | Sulfortransferase family 1A, phenol preferring, member 1 | −2.23 |
| AF052453              | Paps2             | 3'-phosphoadenosine 5'-phosphosulfate synthase 2 | −2.18 |
| X65021                | Gsta3             | Glutathione S-transferase, alpha 3 | −2.16 |
| U16959                | Fkbp5             | FKS06 binding protein 5 | −2.14 |
| AJ132098              | Vnn1              | Vanin 1                  | −2.08 |
| X74351                | Xpa               | Xeroderma pigmentosum, complementation group A | −2.05 |
| M22679                | Adh1              | Alcohol dehydrogenase 1 (class I) | −2.03 |
| AB030836              | Sial7e            | Sialyltransferase 7E | −1.94 |
| M38381                | Clk               | CDC-like kinase         | −1.86 |
| U37091                | Car4              | Carbonic anhydrase 4     | −1.86 |
| AW048113              | Snrk              | SNF-related kinase       | −1.86 |
| AI021125              | Man1a             | Mannosidase 1, alpha     | −1.86 |
| L21671                | Ep8               | Epidermal growth factor receptor pathway substrate 8 | −1.64 |
| AW121182              | Map4k3            | Mitogen-activated protein kinase kinase kinase 3 | −1.64 |
| AA45514               | Abca1             | ATP-binding cassette, subfamily A (ABC1), member 1 | −1.6 |
| U90535                | Fmo5              | Flavin containing monoxygenase 5 | −1.57 |
| L34111                | Idua              | Iduronidase, alpha-1-     | −1.57 |
| D13139                | Dpe1              | Dipeptidase 1 (renal) | −1.56 |
| AW045753              | 1110015E22Rik     | RIKEN cDNA 1110015E22 gene | −1.55 |
| X76479                | Lrp1              | Low-density lipoprotein receptor-related protein 1 | −1.53 |
| AA239571              | Hop1              | Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) | −1.52 |
| X95281                | Rdr1 (pending)    | Retinal short-chain dehydrogenase/reductase 1 | −1.51 |
| M21050                | Lyzs              | Lysozyme                | −1.5 |
| X51547                | Lzp-s             | P lysozyme structural * | −1.47 |
| D50834                | Cyp4b1            | Cytochrome P450, family 4, subfamily h, polypeptide 1 | −1.47 |
| AF020308              | Sfs5              | Splicing factor, arginine/serine-rich 5 (SRp40, HRS) | −1.4 |
| AV376516              | Cyp4b1            | Cytochrome P450, family 4, subfamily h, polypeptide 1 | −1.33 |
| L20590                | Cot3              | Chaperonin subunit 3 (gamma) | 1.23 |
| AW230209              | Mrps18a           | Mitochondrial ribosomal protein S18A * | 1.31 |
| AB025349              | Banf1             | Barrier to autointegration factor 1 | 1.42 |

Continued on following page
| GenBank accession no. | Gene product name | Function or description | Fold change |
|-----------------------|-------------------|-------------------------|-------------|
| AI846849              | Mrps18b           | Mitochondrial ribosomal protein S18B * | 1.43        |
| M26270                | Scd2              | Stearoyl-coenzyme A desaturase 2     | 1.5         |
| X99384                | Pald              | Paladin                   | 1.51        |
| M25358                | Gpd1              | Glycolate-3-phosphate dehydrogenase 1 (soluble) | 1.54        |
| AI840579              | Srr               | Serrine racemase           | 1.57        |
| AA655369              | Timm8a            | Translocase of inner mitochondrial membrane 8 homolog a (yeast) * | 1.59        |
| AW125336              | Pdhb              | Pyruvate dehydrogenase (lipoamide) beta * ** | 1.66        |
| M12330                | Odc               | Ornithine decarboxylase, structural | 1.68        |
| AI080798              | Ptges             | Prostaglandin E synthase      | 1.68        |
| D90374                | Apex1             | Apurinic/apyrimidinic endonuclease 1 | 1.69        |
| AW122260              | Cyp51             | Cytochrome P450, 51           | 1.74        |
| AI847162              | Iysn1             | Myo-inositol 1-phosphate synthase A1 | 1.74        |
| Y11666                | Hk2               | M. musculus gene encoding hexokinase II, exon 1 (and joined CDS) | 1.77        |
| M35970                | Nme1              | Expressed in non-metastatic cells 1, protein (NM23A) (nucleoside diphosphate kinase) | 1.8         |
| AI845854              | Dusp6             | Dual specificity phosphatase 6 | 1.84        |
| M32599                | Gapd              | Glyceraldehyde-3-phosphate dehydrogenase | 1.88        |
| AA716963              | Idi1              | Isopentenyl-diphosphate delta isomerase | 1.91        |
| X17065                | Fkbp4             | FKS06 binding protein 4 | 1.92        |
| AI021127              | Nsdhl             | NAD(P)-dependent steroid dehydrogenase-like | 2.05        |
| X97047                | Pkm2              | Pyruvate kinase, muscle      | 2.07        |
| AW106745              | Nsdhl             | NAD(P)-dependent steroid dehydrogenase-like | 2.09        |
| AI841389              | Eno1              | Enolase 1, alpha nonneuron | 2.12        |
| AI057368              | D scl             | 7-Dehydrocholester reductase | 2.16        |
| AW121639              | Acly              | ATP citrate lyase           | 2.3         |
| AW045533              | Fdps              | Farnesyl diphosphate synthetase | 2.32        |
| J02652                | Mod1              | Malic enzyme, supernatant ** | 2.33        |
| AI846851              | Fdps              | Farnesyl diphosphate synthetase | 2.36        |
| D42408                | S gle             | Squalene epoxidase         | 2.39        |
| AW120625              | Pgdl              | Phosphogluconate dehydrogenase ** | 2.65        |
| AW049778              | Mvd               | Mevalonate (diphosphate) decarboxylase | 3.1        |
| AW122523              | Elov6             | ELOVL family member 6, elongation of long chain fatty acids (yeast) ** | 3.44        |
| AI839004              | Elov6             | ELOVL family member 6, elongation of long chain fatty acids (yeast) ** | 3.63        |

**Cell growth**

| GenBank accession no. | Gene product name | Function or description | Fold change |
|-----------------------|-------------------|-------------------------|-------------|
| AI842277              | Igfbp3            | Insulin-like growth factor binding protein 3 ** | 5.61        |
| X58151                | Igfbp3            | Insulin-like growth factor binding protein 3 ** | 4.3         |
| D13759                | Map3k8            | Mitogen-activated protein kinase kinase kinase 8 | 3.5         |
| X13945                | Lmyc1             | Lung carcinoma v-myc-related oncogene 1 | 3.26        |
| U22399                | Cdkn1c            | Cyclin-dependent kinase inhibitor 1C (P57) | 1.84        |
| U95826                | Ccng2             | Cyclin G2               | 1.76        |
| Z16410                | Btg1              | M. musculus btg1 mRNA | 1.68        |
| AF104010              | Pkd2              | Polycystic kidney disease | 1.45        |
| AF099973              | Slfn2             | Schlafen 2 **            | 1.24        |
| L32751                | Ran               | RAN, member RAS oncogene family | 1.4         |
| AI844810              | Mapk6             | Mitogen-activated protein kinase 6 | 1.55        |
| X95280                | Gb2              | Gbeta/gamma gene 2 | 1.96        |
| M12848                | Myb               | Myeloblastosis oncogene | 2.86        |

**Cell adhesion**

| GenBank accession no. | Gene product name | Function or description | Fold change |
|-----------------------|-------------------|-------------------------|-------------|
| L07803                | Thbs2             | Thrombospondin 2        | 3.37        |
| L19932                | Tgbi              | Transforming growth factor, beta induced * | 1.67        |
| AV375788              | Tnxb              | Tenascin XB *            | 1.57        |
| U43541                | Lamb2             | Laminin, beta 2 *       | 1.49        |
| AI132491              | Bysl              | Bystin-like **           | 2.06        |

**Cell motility and chemotaxis**

| GenBank accession no. | Gene product name | Function or description | Fold change |
|-----------------------|-------------------|-------------------------|-------------|
| AFU30636              | Cxcl13            | Chemokine (C-X-C motif) ligand 13 | 3.67        |
| M70642                | Ctgf              | Connective tissue growth factor * ** | 2.86        |
| U49513                | Cd9               | Chemokine (C-C motif) ligand 9 | 2.18        |
| AB023418              | Cd8               | Chemokine (C-C motif) ligand 8 | 1.95        |

**Response to external stimulus**

| GenBank accession no. | Gene product name | Function or description | Fold change |
|-----------------------|-------------------|-------------------------|-------------|
| M74125                | Ifi205            | Interferon-activated gene 205 | 2.21        |
| M55181                | Penk1             | Preproenkephalin 1 | 2.07        |
| M31312                | Fgfr2b            | Fc receptor, immunoglobulin G, low affinity IIb | 1.7         |
| X06454                | C4                | Complement component 4 (within H-2S) | 1.58        |
| GenBank accession no. | Gene product name | Function or description | Fold change |
|-----------------------|-------------------|-------------------------|-------------|
| AF010254              | Serping1          | Serine (or cysteine) proteinase inhibitor, clade G, member 1 | −1.51       |
| M29009                | Cth               | Complement component factor h                                  | −1.51       |
| X58861                | C1qa              | Complement component 1, q subcomponent, alpha polypeptide * **  | −1.49       |
| D106232               | Ly6c              | Lymphocyte antigen 6 complex, locus C                           | −1.48       |
| M22531                | C1qb              | Complement component 1, q subcomponent, beta polypeptide *      | 1.34        |
| X60676                | Serpinh1          | Serine (or cysteine) proteinase inhibitor, clade H, member 1    | 1.45        |
| U72830                | Stip1             | Stress-induced phosphoprotein 1                                 | 1.54        |
| L40406                | Hsp105            | Heat shock protein                                              | 2.03        |

**Apoptosis**

| M3649                 | Tnfrsf6           | Tumor necrosis factor receptor superfamily, member 6             | −2.91       |
| M1628                 | Fgl2              | Fibrinogen-like protein 2                                       | −1.77       |
| AF041054              | Bnip3             | BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3 *       | −1.68       |
| U21050                | Traf3             | TNF receptor-associated factor 3                                 | 1.43        |
| AV109962              | Traf4             | TNF receptor associated factor 4                                 | 2.22        |

**Signal transduction**

| AB021861              | Map3k6            | Mitogen-activated protein kinase kinase kinase 6                 | −2.82       |
| U70210                | Api2b             | Amyloid beta (A4) precursor protein binding, family B, member 2  | −2.58       |
| AI839138              | Tnrip             | Thioredoxin-interacting protein                                  | −2.48       |
| AI854794              | Tencl             | Tensin 2                                                         | −2.05       |
| D17292                | Admr              | Adrenomedullin receptor                                           | −1.5        |
| AI250490              | Ramp2             | Receptor (calcitonin)-activity-modifying protein 2               | −1.45       |
| U58992                | Madh1             | MAD homolog 1 (Drosophila)                                       | −1.36       |
| AV378405              | Entpd2            | Ectonucleoside triphosphate diphosphohydrolase 2 *              | 1.31        |
| AI842665              | 130001IC24Rik     | RIKEN cDNA 130001IC24 gene                                       | 1.4         |
| U06834                | Ephb4             | Eph receptor B4                                                  | 1.42        |
| U94828                | Rgs16             | Regulator of G-protein signaling 16                              | 1.91        |

**Transport**

| L22218                | Kcnab5            | Potassium voltage-gated channel, shaker-related subfamily, member 5 | −2.43 |
| M24417                | Abhd1a            | ATP-binding cassette, subfamily B (MDR/TAP), member 1A           | −2.24 |
| X82648                | Apod              | Apolipoprotein D                                                 | −2.05 |
| L02914                | Aqp1              | Aquaporin 1 *                                                   | −1.69 |
| L13732                | Scl11a1           | Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 | −1.62 |
| AW124133              | 3010027G13Rik     | RIKEN cDNA 3010027G13 gene                                       | 1.58 |
| L32752                | Rasld-9           | RAS-like, family 2, locus 9                                      | 1.59 |
| AW121088              | Copz1             | Coatomer protein complex, subunit zeta 1                        | 1.64 |
| D55720                | Kpn2a             | Karyopherin (importin) alpha 2                                   | 1.86 |
| AA683883              | Slc25a10          | Solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10 * ** | 1.95 |
| AV218217              | Slc25a1           | Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1 * ** | 1.95 |
| AI848354              | Slc25a1           | Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1 * ** | 2.01 |
| AJ223066              | Fabp5             | Fatty acid binding protein 5, epidermal                          | 2.03 |

**Transcription**

| X61800                | Cespd             | CCAAT/enhancer binding protein (C/EBP), delta **                | −2.91 |
| AF022992              | Per1              | Period homolog 1 (Drosophila)                                   | −2.3 |
| Y12496                | Bteb1             | Basic transcription element binding protein 1                   | −2.2 |
| AF053062              | Nrip1             | Nuclear receptor-interacting protein 1 *                        | −2.18 |
| U62908                | Zfp96             | Zinc finger protein 96                                          | −1.88 |
| AW125783              | Kiif13            | Kruppel-like factor 13 **                                       | −1.7 |
| X57087                | Lyl1              | Lymphoblastic leukemia                                          | −1.61 |
| U60453                | Ezh1              | Enhancer of zeste homolog 1 (Drosophila)                        | −1.53 |
| AI842968              | 2610209L14Rik     | RIKEN cDNA 2610209L14 gene                                       | −1.53 |
| AW047728              | Pcaf              | p300/CBP-associated factor                                       | −1.48 |
| AF020308              | Sfrs5             | Mus musculus HRS gene, complete cds                            | −2.05 |
| U43548                | Tcf4              | Transcription factor-like 4                                     | 2.05 |
| Y07836                | Bhlhb2            | Basic helix-loop-helix domain-containing, class B2              | 3.72 |

**Development**

| X83869                | Nnat              | Neuronatin                                                      | −3.36 |
| X57971                | Gja4              | Gap junction membrane channel protein alpha 4                  | −2.01 |
| AF082567              | Heph              | Hephastin                                                       | −1.82 |

*Continued on following page*
Cyclin D1 governs mitochondrial function and glycolysis in vivo. As gene expression profiling demonstrated that a reduction in cyclin D1 abundance induced expression of nuclear genes encoding mitochondrial function, functional studies of mitochondrial activity were conducted in mammary epithelium from the inducible cyclin D1 antisense transgenic mice. Mammary epithelium from the ponasterone-inducible cyclin D1 antisense-ErbB2 transgenic mice was introduced into primary culture. Culture of the mammary epithelium in the absence of stroma was conducted to determine whether cyclin D1 expression in the mammary epithelium was sufficient for the alterations in mitochondrial function observed in vivo. Real-time RT-PCR analysis demonstrated the reduction in cyclin D1 mRNA, induction of cyclin D1 antisense mRNA, and the induction of β-galactosidase (Fig. 6A). Induction of cyclin D1 antisense in the transgenic mammary epithelium in culture reduced cyclin D1 protein abundance (Fig. 6A). As a measure of mitochondrial function, MitoTracker analysis was conducted. MitoTracker Red CMXROS was used to stain functioning mitochondria in living cells, yielding a fluorescent signal whose intensity is dependent on mitochondrial membrane potential and mass (44). Induction of cyclin D1 antisense in transgenic mammary epithelium increased MitoTracker activity by 61% (Fig. 6B and C).

To determine whether the regulation of mitochondrial function by cyclin D1 was specific to untransformed murine mammary epithelium, transformed human MCF7 cells and a cell line derived from MMTV-ErbB2 transgenic mice (NAFA) were assessed (Fig. 7). Retroviral transduction of the murine cyclin D1 antisense into the MMTV-ErbB2 mammary tumor-derived cell line NAFA resulted in a 75% reduction in cyclin D1 protein abundance, an 80% reduction in cyclin D1 mRNA, and a 47% increase in MitoTracker activity (Fig. 7A through C). Cyclin D1 siRNA reduced cyclin D1 protein levels 80% in MCF7 cells and increased MitoTracker activity 41% (Fig. 7D and E). Multiple independent studies using cyclin D1 siRNA showed identical reductions in cyclin D1 abundance and inductions of increased MitoTracker activity. A time course effect was determined using siRNA to cyclin D1. MCF7 cells were treated with 600 nM siRNA for time periods from 6 to 60 h. Associated with the reduction in cyclin D1 abundance, MitoTracker activity increased from 30% to 40% between 36 and 60 h (Fig. 7F and G). Consistent with an increase in hexokinase activity observed in cyclin D1 antisense-transduced cells, cyclin D1 siRNA treatment of MCF7 cells for 12 h increased hexokinase II activity from 10 to 22 pmol/µg/min (Fig. 7H). To complement MitoTracker Red CMXROS analysis of mitochondrial activity, we also conducted JC-1 staining. Within the cell, JC-1 exists mainly in a monomeric form which emits green fluorescence. The intensity of JC-1 green fluorescence

| GenBank accession no. | Gene product name | Function or description | Fold change $^b$ |
|-----------------------|-------------------|-------------------------|-----------------|
| AW050256              | Tubb3             | Tubulin, beta 3          | 1.55            |
| AW215756              | 2310057H16Rik     | RIKEN cDNA 2310057H16 gene | 1.63            |
| X04663                | Tubb5             | Tubulin, beta 5          | 1.68            |
| L39017                | Procr             | Protein C receptor, endothelial | -1.97          |
| L24739                | Pros1             | Protein S (alpha)        | -1.49           |
| AF045887              | Agt               | Angiotensinogen          | -2.9            |

$^a$ Gene function or description is used to subcategorize genes altered in expression to include mitochondrial genes and genes involved in metabolism and extracellular functions. $^*$, duplicated genes; **, genes regulated reciprocally in MMTV-cyclin D1 mammary tumors.

$^b$ Mean fold change in expression, assessed by Affymetrix microarray.

TABLE 3—Continued
FIG. 4. Cyclin D1 antisense-regulated genes in vivo. A schematic representation is shown of gene products regulating mitochondrial function and lipogenesis. Genes that are induced by mammary epithelial cell-targeted cyclin D1 antisense, assessed by gene expression profiling, are shown in red arrows (↑); those repressed are shown in blue arrows (↓). **, genes regulated reciprocally in MMTV-cyclin D1 mammary tumors. TCA, tricarboxylic acid; FAD, flavin adenine dinucleotide; FADH₂, reduced FAD; DHCR, 7-dehydrocholesterol reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase.
FIG. 5. Molecular signature from cyclin D1-induced mammary epithelial tumors—inhibition of lipogenesis, glycolysis, and mitochondrial gene function. (A) Treeview analysis of microarray expression data comparing mammary tumors derived from MMTV-cyclin D1 transgenic mice showing genes regulated more than twofold. Levels for expression are shown for either up-regulated genes (red) or down-regulated genes (green). See http://www.jci.tju.edu/pestell/papers/CD1AS (worksheet labeled “31 genes”) for the gene accession numbers and names. Genes that are regulated reciprocally to mammary epithelial cell-targeted cyclin D1 antisense are indicated by gene names in red with the gene product names in parentheses. These genes regulate mitochondrial metabolism and lipogenesis. (B) Pearson correlation coefficient analysis. Separation of gene groups by metagene analysis is shown for transgenic mice treated with either ponasterone A, placebo, or MMTV-cyclin D1 tumors.
has previously been used as an indication of mitochondrial mass (38). In cyclin D1 siRNA-treated MCF7 cells, green fluorescence was increased 42% compared to control cells. As cyclin D1 encodes a key component of the holoenzyme that phosphorylates pRb, we applied JC-1 staining to measure mitochondrial mass (green fluorescence) in fibroblasts derived from mice deleted of either the regulatory (cyclin D1) or catalytic (Cdk4) component of the holoenzyme. Relative to wild-type fibroblasts, JC-1 staining was increased in the cyclin D1−/− (199%) and cdk4−/− (6%) fibroblasts relative to wild-type MEFs.

Genes promoting oxidative glycolysis, lipogenesis, and mitochondrial function were induced in the mammary epithelium upon induction of cyclin D1 antisense in transgenic mice. Assessment of cyclin D1−/− mammary epithelial cells by Western blotting demonstrated a similar induction of the key regulatory proteins of oxidative glycolysis (hexokinase II, pyruvate kinase) and fatty acid synthesis (fatty acid synthase, acetyl-CoA oxidase) (Fig. 8A). In view of the induction of nuclear genes regulating mitochondrial function, transmission electron microscopy was conducted on mammary epithelial cells from the mammary gland of cyclin D1−/− and cyclin D1 wild-type (wt) mice (Fig. 8B). Mammary epithelial cell mitochondria were increased three- to fourfold in size in cyclin D1−/− mice compared with cyclin D1 wt mice (cyclin D1+/+ versus cyclin D1−/−, P < 0.05). In mammary epithelial cells the mitochondria of wt (2.5 μm × 1.6 μm) were significantly smaller than those of cyclin D1−/− (7.8 μm × 2.6 μm) by stereoscopy. The relative volume fraction of the mitochondria within the cell was approximately 6% in the wild-type mammary epithelial cells and 16% in the cyclin D1−/− cells.

As an in vivo measurement of relative utilization of amino acids from the tricarboxylic acid cycle, the ratio of (glutamate + glutamine)/citrate was assessed (38) using nuclear magnetic resonance. Mammary gland spectroscopy was conducted on 3-month-old female cyclin D1−/− mice, with comparison made to littermate control wt females of the same age (Fig. 8C). In cyclin D1−/− mice, mammary gland (glutamate + glutamine)/citrate was increased (1.8 [±0.7]-fold). Similar results to those of the cyclin D1−/− mice were observed upon induction of the cyclin D1 antisense transgene, comparing ponasterone A-treated and placebo-treated ErbB2-cyclin D1 antisense mice (2.3 [± 0.5]-fold, n = 4). Together, these studies suggest the increased abundance of cyclin D1 determines metabolic substrate prioritization toward amino acid synthesis from the tricarboxylic acid cycle, consistent with the known role for cyclin D1 in DNA synthesis.

**DISCUSSION**

The functional analysis of the molecular phenotype identified herein demonstrated that endogenous cyclin D1 normally inhibits oxidative glycolysis, lipogenesis, and mitochondrial gene activity in the mammary epithelium in vivo. Global gene expression profiling has been used to identify biologically relevant molecular signatures, histological subtypes, and prognostic features (20, 45, 63) in human cancer. Reproducible patterns of gene expression are altered in a selective manner during the tumorigenic process. Cyclin D1 is overexpressed early in tumorigenesis and is thus coexpressed with a number of genes that are sequentially engaged during tumor progression. Because cyclin D1 is relatively ubiquitously expressed, regulates normal mammary gland development, and contributes to the normal function of cells (including blood vessels, adipocytes, and bone marrow macrophages) (5, 47, 58), which in turn contribute to normal breast development and tumorigenesis, dissection of the molecular genetic targets of cyclin D1 in vivo requires mammary epithelial cell-type targeted regulated gene expression in the immune-competent animal. Employing tissue-specific inducible cyclin D1 antisense transgens and gene expression profiling, we have determined the global gene expression profile coordinated by cyclin D1 in the mammary epithelial cell.

Cyclin D1, which is frequently overexpressed in human breast tumors and in ductal carcinoma in situ (1, 25, 41, 59), functions in several distinct molecular complexes/pathways (42, 62). As a regulatory subunit of a holoenzyme that phosphorylates pRb, cyclin D1 inactivates the G1 checkpoint function for pRb, sequentially inducing E2F-responsive genes (46, 50). Through sequestration of p27kip1 and p21cip1/waf1, cyclin D1 can enhance cyclic E/CDK2 kinase activity (46, 50). Finally, cyclin D1 regulates several transcriptional targets, including the estrogen receptor, the androgen receptor, v-Myb, DMP1, C/EBPβ, and PPARγ (24, 66) in a CDK-independent manner, in part through repressing the coactivator p300 (59) and through recruitment of HDACs (19). Mammary epithelial cell-targeted inducible cyclin D1 antisense transgens are predicted to inactivate both CDK-dependent and -independent functions and therefore offer a comprehensive analysis of in vivo cyclin D1 genetic targets. E2F-responsive genes and genes induced by DNA synthesis were identified herein. The induction by cyclin D1 antisense of lipogenic genes in mammary epithelium in vivo (Fig. 4 and Table 1) is consistent with the hepatic steatosis described in cyclin D1−/− mice. Key regulators of adipogenesis include PPARγ, C/EBPβ, and pRb, each of which has been described as a target of cyclin D1. The increase in fatty acid synthesis driven by increased malonyl CoA and acetyl-CoA carboxylase is observed upon induction of AMP-activated protein kinase upon glucose deprivation and, like cyclin D1 antisense, is associated with a reduction in cellular proliferation (17). The induction of lipogenic genes by cyclin D1 antisense is consistent with the known physiological role for cyclin D1 as an inhibitor of lipogenesis (29).

Cyclin D1-dependent inhibition of mitochondrial activity was demonstrated by microarray analysis and confirmed by functional assays of MitoTracker activity in the cyclin D1 antisense mammary epithelium. Genome-wide expression studies of cyclin D1 antisense demonstrated induction of mitochondrial metabolism. Antisense cyclin D1, siRNA, and genetic-knockout cells demonstrated that physiological levels of cyclin D1 expression normally inhibit mitochondrial activity. This function of cyclin D1 was conserved in normal, immortalized, and transformed mammary epithelial cells. Scanning electron microscopy confirmed the increased size of mitochondria in cyclin D1−/− cells. Thus, cyclin D1 serves dual functions: to promote nuclear DNA synthesis and to inhibit mitochondrial activity. Considered as bacterial ancestors endosymbiotically integrated into the eukaryotic cytoplasm, mitochondria serve as key regulators of diverse cellular functions. Cyclin D1 is a labile growth factor- and oncogene-inducible protein and as such may serve to integrate growth factor signals to energy and biosynthetic priorities (59).
FIG. 6. Cyclin D1 antisense enhances mammary epithelial cell mitochondrial activity. (A) The primary cultures of mammary epithelium from cyclin D1 antisense-ErbB2 transgenic mice were treated with either vehicle (−) or ponasterone A (+) (10 μM) for 48 h and assessed by (left) real-time RT-PCR or (right) Western blotting. (B) MitoTracker activity of cyclin D1 antisense-ErbB2 transgenic epithelium versus “ErbB2 control line” transgenic mice (Fig. 2A) assessed in situ and quantitated by fluorescence-activated cell sorter analysis (C). DDCt, ΔΔ cycle threshold; FL2, filter that detects fluorescence at 599 nm; Pon A, ponasterone A.
FIG. 7. Cyclin D1 inhibits breast epithelial tumor cell mitochondrial activity. (A) The NAFA cell line infected with the cyclin D1 antisense retrovirus vector (mouse stem cell virus-cyclin D1 antisense-IREs-GFP) was analyzed by Western blotting for cyclin D1 with GDI used as a loading control for protein. (B) Northern blot analysis for cyclin D1 sense and anti-sense mRNA. (C) The relative change in MitoTracker activity (shown for representative experiments for \( n = 3 \)). (D) Western blot analysis of MCF7 cells 72 h after transduction with control or cyclin D1 siRNA. (E) Mitochondrial activity determined by MitoTracker at 72 h. (F) Western blot time course analysis of MCF7 cells after transduction with control or cyclin D1 siRNA and (G) corresponding MitoTracker activity. (H) Hexokinase activity of MCF7 cells treated with cyclin D1 siRNA. FL2, filter that detects fluorescence at 599 nm.
FIG. 8. Cyclin D1 deletion increases breast epithelial cell expression of proteins regulating glycolysis, lipogenesis, and mitochondrial size. (A) Western blot analysis of mammary epithelium from wt or cyclin D1−/− mice with antibodies for the indicated proteins. Fatty acid synthase (FAS) (lipogenic gene), acetyl-CoA carboxylase (ACC), hexokinase II (HK II) (glycolytic gene), pyruvate kinase (PK) and a control for loading (GDI) are shown. (B) Transmission electron microscopic images of mammary epithelial cells of wt or cyclin D1−/− mice, showing increased mitochondrial size in cyclin D1−/− mice (scale bar = 1 μm). N, nucleus; arrows, mitochondria. (C) Mouse mammary gland spectroscopy. Thin-line spectra were obtained from cyclin D1−/− mouse mammary gland in vivo. Bold-line spectra were obtained from normal mouse mammary gland in vivo.
Cyclin D1 antisense expression in transgenic mice reduced cyclin D1 abundance and induced oxidative glycolysis, evidence by altered gene expression, protein abundance, and enzyme activity. The induction of oxidative glycolysis was evidenced by the induction of pyruvate kinase and hexokinase II. Nuclear magnetic resonance of the mammary glands of cyclin D1−/− mice and cyclin D1 wt mice showed increased (glutamate + glutamine)/citrate in cyclin D1−/− compared with cyclin D1 wt mice (Fig. 5C), suggesting mammary epithelial cell cyclin D1 regulates metabolism within the whole mammary gland. An elevation of (glutamate + glutamine)/citrate is consistent with a reduction in utilization of these precursors of DNA synthesis. The role of hexokinase II in cellular growth, senescence, and survival is complex and depends upon environmental cues (18, 23, 51). Hexokinase II was reduced in cyclin D1-overexpressing and ErbB2-overexpressing tumors but not in c-Myc-overexpressing tumors. The increase in 6-phosphogluconate dehydrogenase in the cyclin D1 antisense epithelium is a marker of increased shunting of available glucose from the glycolytic pathway to the pentose phosphate shunt and is observed in replicative cellular senescence and proposed to be a tumor suppressor mechanism in response to bioenergetic stress (16).

The mammary tumors induced by cyclin D1 and the cyclin D1 antisense arrays demonstrated reciprocal expression of genes involved in mitochondrial metabolism. The enhanced mitochondrial activity of cyclin D1 antisense-expressing cells herein and the corresponding inhibition of tumor growth (7) suggest that these two activities may be linked. High fasting serum glucose and increased IGFBP-3 is associated with high breast cancer risk (35), consistent with the increased IGFBP-3 observed in the MMTV-cyclin D1 mammary tumors, the reduction of mitochondrial activity of cyclin D1 antisense-expressing cells and the corresponding inhibition of tumor growth (7).

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