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Cdc6 and Cyclin E2 Are PTEN-Regulated Genes Associated with Human Prostate Cancer Metastasis[1]

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Abstract

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is frequently inactivated in metastatic prostate cancer, yet the molecular consequences of this and their association with the metastatic phenotype are incompletely understood. We performed transcriptomic analysis and identified genes altered by conditional PTEN reexpression in C4-2, a human metastatic prostate cancer cell line with inactive PTEN. PTEN-regulated genes were disproportionately represented among genes altered in human prostate cancer progression and metastasis but not among those associated with tumorigenesis. From the former set, we identified two novel putative PTEN targets, cdc6 and cyclin E2, which were overexpressed in metastatic human prostate cancer and up-regulated as a function of PTEN depletion in poorly metastatic DU145 human prostate cancer cells harboring a wild type PTEN. Inhibition of cdc6 and cyclin E2 levels as a consequence of PTEN expression was associated with cell cycle G_1 arrest, whereas use of PTEN activity mutants revealed that regulation of these genes was dependent on PTEN lipid phosphatase activity. Computational and promoter-reporter evaluations implicated the E2F transcription factor in PTEN regulation of cdc6 and cyclin E2 expression. Our results suggest a hypothetical model whereby PTEN loss up-regulates cell cycle genes such as cdc6 and cyclin E2 that in turn promote metastatic colonization at distant sites.

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Introduction

Various mechanisms contribute to prostate cancer progression to advanced stage [1,2]. Among them, the deletion of tumor suppressor gene *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) is a frequent molecular alteration associated with tumor progression [3,4]. Although only approximately 30% of primary prostate cancers have PTEN mutations, these are found in up to 60% of metastatic and advanced prostate cancers [5,6]. PTEN deletion is also correlated with a high Gleason score and advanced tumor stage [7]. Although the correlation between loss of PTEN activity and prostate cancer progression is clear, the effectors responsible for this association remain incompletely characterized.

Genome-wide expression analysis of human tumors has demonstrated that models, which predict clinical outcome in patients, can be constructed [8]. Gene expression signatures can also be identified, which reflect the activation status of oncogenic pathways, and these in turn provide clinically relevant associations with disease outcomes [9]. Recently, a microarray gene expression signature for immunohistochemistry (IHC)-detectable PTEN loss in breast cancer has been developed [10]. Importantly, some PTEN IHC-positive cancers exhibited the signature of PTEN loss, suggesting that the signature is more sensitive than PTEN IHC for identifying tumors with pathway activation. Furthermore, this signature correlated to poor patient outcome in independent data sets of breast, bladder, and prostate carcinoma.

With the objective of determining effectors of PTEN that are related to prostate cancer metastasis, here we use a conditional PTEN reexpression model [11] to develop gene expression signatures associated

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with PTEN. To identify PTEN-regulated genes important in prostate cancer progression and metastasis, we focused on transcripts whose expression was altered as function of human prostate cancer metastasis. Using this approach, we identified cdc6 and cyclin E2 as novel PTEN-regulated targets associated with prostate cancer metastasis.

Materials and Methods

Reagents

Akt inhibitor, 1,3-dihydro-1-(1-((4-(6-phenyl-1*H*-imidazo[4,5-*g*]quinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2*H*-benzimidazol-2-one (Akt inhibitor VIII), and JNK inhibitor, anthra[1,9-*cd*]pyrazol-6(2*H*)-one (SP600125), were purchased from EMD Chemicals (San Diego, CA).

Transcriptional Analysis of pTetOn PTEN C4-2 Cells

pTetOn PTEN C4-2 prostate cancer cells in which expression of PTEN is under the control of TetOn system were previously described [11]. Cells were treated with 1 μg/ml doxycycline (DOX) or vehicle for 24 hours and then total RNA were isolated and hybridized to HG-U133A GeneChip Array (Affymetrix, Santa Clara, CA) as described [12]. Cells used for transcriptional profiling were concurrently evaluated by Western blot to confirm expression of the *PTEN* transgene as described [11]. This experiment was repeated with a completely different batch of cells 1 week after the initial experiment to provide duplicate samples for statistical analysis. The local pooled error (LPE) test [13] was used to find differentially altered probe sets in response to DOX treatment (PTEN expression). Probe sets with a false discovery rates (FDRs) of <.01 were designated as regulated by PTEN.

Unsupervised Cluster Analysis of Probe Sets Altered by PTEN Expression as Compared to Probe Sets in Human Prostate Cancer Tumors

Using RMA [14] implemented in the Bioconductor project (http://www.bioconductor.org/), we computed expression measures from PTEN and human prostate tumor microarray data [15]. Details of human tumor samples were described before [15]. Log_2 transformation and interquartile range normalization were carried out for both data sets. Because these two sets were hybridized to different platforms (U133Plus2 for human tumor set and U133A for PTEN/cell lines set), we compared UniGene IDs between these two data sets and selected only probe sets in the human data that matched those identified as PTEN-regulated in the analysis described above. Hierarchical clustering analysis was performed on the human data with those selected probe sets because it clusters coexpressed genes and shows expression patterns of gene clusters effectively. Thus, all the probe sets that were clustered on the human data were also specifically regulated by PTEN expression with statistical significance based on the LPE test.

To further explore the correlation of PTEN-regulated probe sets with those altered as a function of prostate cancer metastasis, the number of probe sets that were differentially regulated by PTEN was reduced by examining the differential expression of correlated probe sets in the human data set between metastatic and nonmetastatic samples. Using the two-sample *t* tests, we examined the statistical significance between benign and localized samples and the significance between localized and metastatic samples. After computing Benjamini and Hochberg–adjusted *P* values [16] from the raw *P* values of the two-sample *t* tests using the multitest package of the Bioconductor project because of the multiple testing, we selected probe sets that met both criteria, adjusted *P* values >.05 (no statistical difference) between the benign and localized samples and the adjusted *P* values <.05 (statistical different) between the localized and metastatic samples. In other words, the expression levels of those selected probes are statistically different between localized and metastatic samples, whereas their expression levels were similar between benign and localized samples. Hierarchical clustering analysis was performed on this subset of probe sets as described above.

Promoter Analysis and Interaction Network Toolset Analysis

To find putative transcription factors associated with gene expression changes altered by PTEN expression, we used the Promoter Analysis and Interaction Network Toolset (PAINT) [17] (http://www.dbi.tju.edu/dbi/tools/paint/). This tool identifies and quantitates overrepresentation of transcription factor binding sites in the promoters of PTEN-regulated gene sets. Gene accession numbers corresponding to statistically significant (FDR < .01) PTEN-regulated probe sets were uploaded into PAINT. TRANSFAC Public database was chosen to find transcriptional regulatory elements (TREs) on upstream gene sequences. Enriched TREs were identified by comparing promoter regions of genes down-regulated by PTEN to all human promoter sequences in the PAINT database.

Real-time Reverse Transcription–Polymerase Chain Reaction Analysis of Cdc6 and Cyclin E2 Expression Levels After PTEN Reexpression

Real-time reverse transcription–polymerase chain reaction (RT-PCR) was carried out on iCycler Optical Module (Bio-Rad, Hercules, CA) with IQ SYBR Green (Bio-Rad) fluorescent dye included in the PCR to determine the amount of mRNA level for cdc6 and cyclin E2. Primers used for cdc6 were forward, 5′-GAGATGTTCGCAAAGCACTG-3′, and reverse, 5′-TGGGAATCAGAGGCTCAGA-3′. Primers for cyclin E2 were forward, 5′-CTATTTGGCTATGCTGGAGG-3′, and reverse, 5′-TCTTCGGTGGTGTCATAATG-3′. Real-time RT-PCR for glucuronidase-β was used as an internal control, and primers were forward, 5′-CCGACTTCTCTGACAACCGACG-3′ and reverse, 5′-AGCCGACAAAATGCCGCAGACG-3′. Expression levels of cdc6 and cyclin E2 were normalized to the expression of glucuronidase-β in each sample.

Small Interfering RNA

PTEN small interfering RNA (siRNA) duplex was described previously [18] and was purchased from Dharmacon (Lafayette, CO) with the following target sequence: 5′-CCAGUCAGAGGCGCUAUGU-3′. siRNA duplex targeting luciferase (GL2), 5′-CGTACGCGGAATACTTCGA-3′ was used as control siRNA. Transfection of DU145 human prostate cancer cells (American Type Culture Collection, Manassas, VA) with siRNA duplexes (200 nM) was done in six-well plates using OligofectAMINE (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Cell Cycle Analysis

Cells were trypsinized, and cell cycle analysis with FACSCalibur was carried out [11]. All flow cytometric measurements were done using the same instrument settings, and >10,000 cells were analyzed in each sample. The percentage of cells in different stages of the cell cycle was determined with ModFit LT3.1 software (Verity Software House, Topsham, ME).

DNA Constructs, Transient Transfection, and Luciferase Reporter Assay

Wild type and E2F binding site–mutated cdc6 promoter fragments in pGL3 basic were gifts from Dr. R.S. Williams [19]. The E2F luciferase reporter construct was a gift from Dr. S. Chellappan at University of South Florida. DNA transfection was performed using lipofectin according to the manufacturer's protocol (Invitrogen), and the transfection efficiency was approximately 70% as examined under the fluorescence microscope with a cotransfected green fluorescent protein expression construct. pTetOn PTEN C4-2 cells were transfected, and 24 hours later, 1 μg/ml DOX was added followed by an additional 24 hours of incubation. Cells were lysed and analyzed for luciferase activity according to the manufacturer's protocol (Promega, Madison, WI). Luciferase activity was corrected for cell number with SYBRGold assay (Invitrogen).

Western Blot Analysis

Western blot was carried out as described [11] using anti-HA monoclonal antibody (Covance, Berkeley, CA) at a 1:3000 dilution. The monoclonal anti-cdc6 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a 1:1000 dilution. The polyclonal anti–phospho-Akt (Ser473), anti-Akt, anti–phospho-c-Jun, anti–phospho-Rb, anti–E2F-1, anti-PTEN, and anti–cyclin E2 antibodies were from New England Biolabs (Beverly, MA) and used at a 1:1000 dilution. The monoclonal anti–α-tubulin antibody was purchased from Oncogene (San Diego, CA) and was diluted at 1:2000. The HRP-conjugated antimouse (Pierce Biotechnology, Rockford, IL) or antirabbit (New England Biolabs) secondary antibody was used at a 1:200,000 dilution.

Results

Transcriptional Profiling of pTetOn PTEN(wt) C4-2 as a Function of PTEN Reexpression

Transcriptional profiling was carried out as a function of PTEN reexpression in pTetOn PTEN C4-2 prostate cancer cells [11] (Figure 1). A decrease in phospho-Akt level with the PTEN induction in the C4-2 cells used in profiling was observed, confirming the biochemical impact of the induction. Using the LPE test, we found that 123 probe sets were differentially altered by PTEN expression with an FDR P value <.01. Among those 123 probe sets, 88 were up-regulated after PTEN expression, whereas 35 were down-regulated. The top 10 up- and down-regulated probe sets and corresponding genes are listed in Table 1.

Probe Sets Altered by PTEN Expression Preferentially Cluster with Metastatic Disease in Patients

Because functional loss of PTEN correlates with tumor grade and stage in prostate cancer, we sought to examine whether probe sets altered in response to PTEN reexpression were related to human prostate cancer progression and metastasis. To do this, we matched our PTEN-regulated probe sets to probe sets in a human prostate tumor array set [15] by comparing UniGene IDs because the two array data used different platforms. A total of 388 probe sets in the human tumor

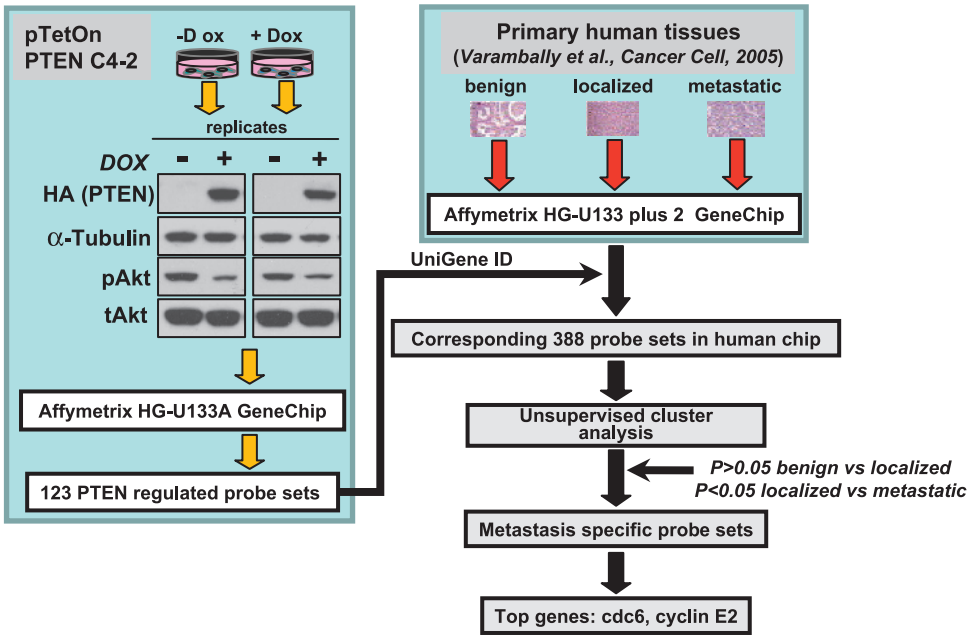

Figure 1. Flow chart of experimental strategy. Gene expression changes in response to PTEN expression were analyzed by Affymetrix GeneChip in pTetOn PTEN C4-2 cells after DOX treatment to reexpress PTEN. Probe sets specifically altered by PTEN were identified, and probe sets in human prostate cancer array data corresponding to PTEN-altered probe sets in C4-2 cells were determined by UniGene IDs. These PTEN-regulated probe sets in human samples were used for hierarchical clustering analysis with human tumor array data. A subset of probes was identified as a function of their altered expression in metastasis but not transformation using a two-sample t test. PTEN target genes, *cdc6* and *cyclin E2*, were further characterized from this subset of probe sets. Western blot confirmed PTEN expression in samples that were identical to those used in Affymetrix chip analysis. Two separate experiments with different batch of cells were evaluated.

Table 1. Top Up- or Down-regulated Genes After PTEN Expression in pTetOn PTEN C4-2 Prostate Cancer Cells.

Probe Set*	Gene Name	Fold Difference†
PTEN up-regulated genes		
213350_at	Ribosomal protein S11	4.237
201427_s_at	selenoprotein P, plasma, 1	3.462
209815_at	patched homolog (*Drosophila*)	2.468
213736_at	Cytochrome *c* oxidase subunit Vb	2.248
202028_s_at	ribosomal protein L38	2.177
212952_at	Transcribed locus	2.003
217989_at	Hydroxysteroid (17-beta) dehydrogenase 11	1.938
204663_at	malic enzyme 3, NADP(+)-dependent, mitochondrial	1.899
202627_s_at	serpin peptidase inhibitor, clade E, member 1	1.899
213110_s_at	collagen, type IV, alpha 5 (Alport syndrome)	1.866
PTEN down-regulated genes		
201490_s_at	peptidylprolyl isomerase F (cyclophilin F)	−2.055
211450_s_at	mutS homolog 6 (*Escherichia coli*)	−1.990
220651_s_at	MCM10 minichromosome maintenance deficient 10 (*Saccharomyces cerevisiae*)	−1.896
213872_at	Chromosome 6 open reading frame 62	−1.857
214079_at	dehydrogenase/reductase (SDR family) member 2	−1.850
203968_s_at	CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	−1.843
211814_s_at	cyclin E2	−1.715
206752_s_at	DNA fragmentation factor, 40 kDa, beta polypeptide (caspase-activated DNase)	−1.696
209572_s_at	embryonic ectoderm development	−1.663
217294_s_at	enolase 1 (alpha)	−1.660

*Affymetrix (www.Affymetrix.com).
†Fold change comparing PTEN-expressing cells (DOX-treated) with PTEN-unexpressed cells (DMSO-treated).

data set were found to correspond to the 123 (88 up- and 35 down-regulated) PTEN-responsive probe sets, respectively. Of the 388, 292 matched to the 88 and 96 to the 35 probe sets in the pTetOn PTEN C4-2 cells. Unsupervised hierarchical clustering of the 388 probe sets was carried out in the human tissue data set to determine how PTEN-regulated genes were associated to prostate cancer stage. Figure 2, *A* and *B*, shows a striking pattern of clustering associated with the transition between localized and metastatic human cancer rather than between benign and malignant disease. This was true for both up- and down-regulated probe sets.

Identification of Probe Sets Altered by PTEN Expression and Associated with Prostate Cancer Metastasis

The data previously mentioned suggest that probes differentially expressed as a function of PTEN expression were more likely to be associated with tumor progression than transformation. To identify candidate metastasis genes (promoters or suppressors) that are regulated by PTEN, we applied a two-sample *t* test on the expression level of each probe identified above in three types of samples (benign, localized, and metastatic) of the human prostate tissue data. Subsets of PTEN-altered probe sets and corresponding genes (up- and down-regulated) were thus identified as differentially expressed between localized and metastatic samples ($P < .05$), whereas their expression levels were not altered between benign and localized samples ($P > .05$). To evaluate the effectiveness of this approach, a hierarchical clustering analysis was applied to these subsets of PTEN-altered probes on the human tumor data. As shown in Figure 2, *C* and *D*, the clustering pattern of these probe sets even more profoundly distinguished metastatic samples from benign and localized samples than before selection (Figure 2, *A* and *B*).

PTEN Inhibits the Expression of Cdc6 *and* Cyclin E2*, Two Genes Up-regulated in Prostate Cancer Metastasis*

For further study, we selected genes that were both among the top 10 PTEN-regulated probe sets and altered as a function of human prostate cancer metastasis. Only two PTEN down-regulated probe sets met this criterion, corresponding to cdc6 and cyclin E2. Examination of the human prostate cancer array data [15] showed higher expression levels of all cdc6 and cyclin E2 probe sets in metastatic samples compared to benign and localized samples (Figure 3, *A* and *B*). Elevated expression levels of cdc6 and cyclin E2 in metastatic samples were further supported by results of independent analysis using Oncomine (Figure 3, *C* and *D*) [20].

To validate our microarray data, we used real-time RT-PCR to examine expression levels of cdc6 and cyclin E2 in response to PTEN induction and found that their expression levels were inhibited approximately four- and three-fold, respectively (Figure 4*A*), confirming the PTEN chip result. Consistent with mRNA levels, the protein levels of cdc6 and cyclin E2 were also decreased by three- and two-fold, respectively, after PTEN expression as indicated by Western blots (Figure 4*B*). Real-time RT-PCR was also carried out on pTetOn C4-2 cells that lacked pTRE2-PTEN vector exposed to DOX. No significant change of *cdc6* and *cyclin E2* expression levels was observed in these cells (data not shown), indicating that the inhibition of *cdc6* and *cyclin E2* expression was specific to PTEN induction. To independently verify the regulatory effect of endogenous PTEN on these two genes, we depleted this protein in DU145, a poorly metastatic human prostate cell line with high PTEN expression levels [11]. In support of PTEN regulation of cdc6 and cyclin E2 expression, depletion of this former protein (Figure 4*C*) increased the expression of the latter two (Figure 4*D*).

Inhibition of Cdc6 and Cyclin E2 Expression After PTEN Induction Correlated with a Cell Cycle G_1 Block

Both cdc6 and cyclin E2 are implicated in the cell cycle G_1- to S-phase transition [19,21], and our previous work showed that PTEN expression blocked C4-2 cells at the G_1 phase [11]. We began evaluating the relationship of PTEN to cdc6 and cyclin E2 by examining the correlation of cdc6 and cyclin E2 expression with cell cycle G_1 arrest after PTEN reexpression. Minor expression of PTEN was

observed after 12 hours of treatment with DOX, and its expression reached maximal after 24 hours of DOX treatment (Figure 5*A*). Significant cell cycle G_1 arrest was not observed until 24 hours of treatment with DOX, with the percentage of cells at the G_1-phase increasing from 54% to 75% (Figure 5*B*). The expression levels of cyclin E2 and cdc6 were significantly inhibited after 12 hours of treatment with DOX, and both expression levels were maximally reduced at 24 hours of DOX treatment (Figure 5*C*). The correlated cell cycle G_1 block with reduced expression of cyclin E2 and cdc6 after PTEN expression suggests that the cell cycle G_1 arrest after PTEN expression in C4-2 cells may be associated with PTEN inhibition in cyclin E2 and cdc6 expression.

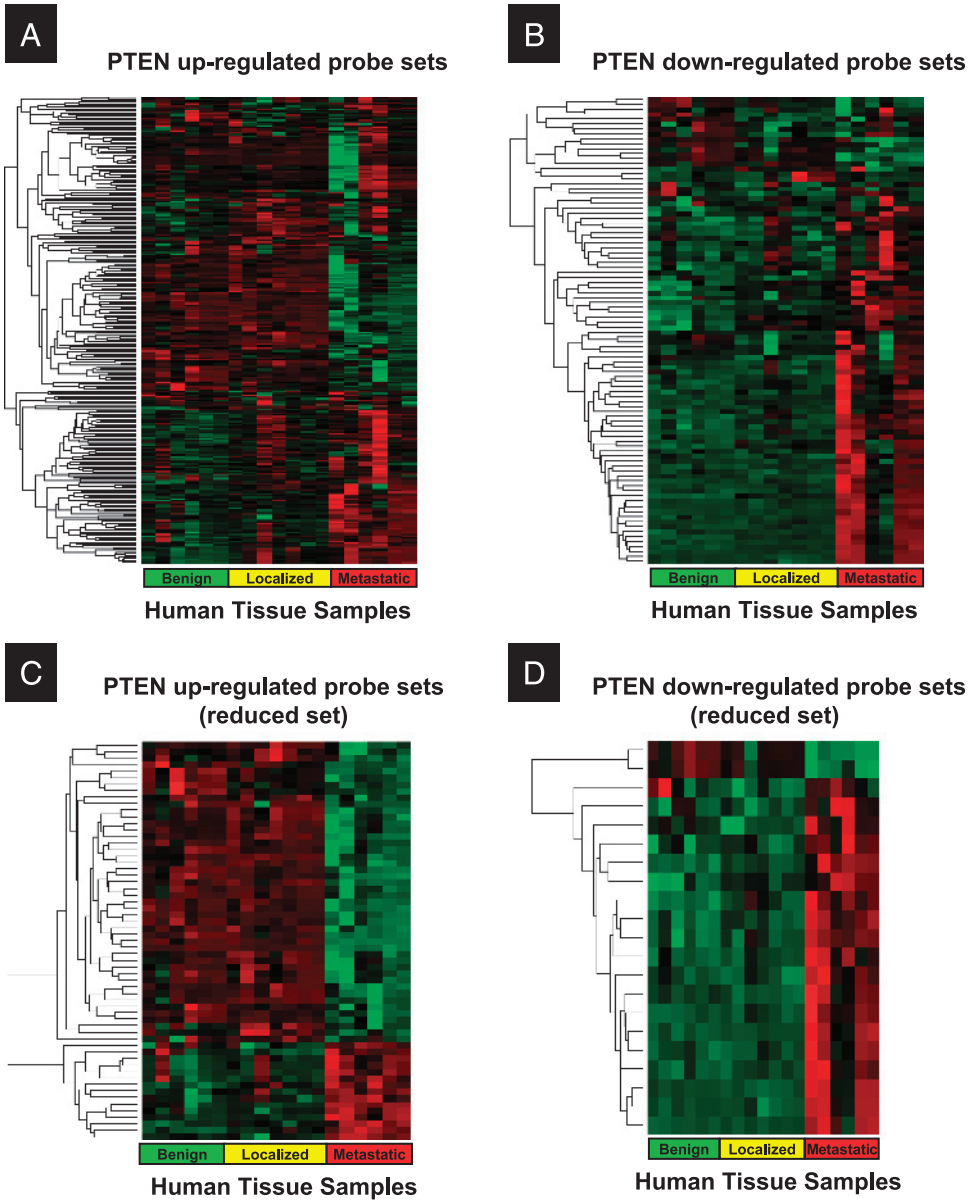

Figure 2. (A–B) Unsupervised cluster analysis of all probe sets regulated by PTEN in C4-2 cells, which matched with those in human prostate tumor array data. Probe sets that are specifically regulated by PTEN in our pTetOn PTEN C4-2 chip analysis were identified and linked to probe sets in human tumor array data based on their UniGene IDs. A total of 292 (A) and 96 (B) probe sets in human array data were found to be corresponding to 88 up- and 35 down-regulated PTEN-responsive probe sets, respectively. These 96 and 292 probe sets were used in an unsupervised hierarchical cluster analysis with human tumor array data, and heat maps were generated to show the expression patterns of these probe sets among the three sample sets (benign, localized, and metastatic) of prostate cancer tissues. (C–D) Cluster analysis of a subset of PTEN-regulated probe sets as a function of prostate cancer metastasis. The number of probe sets (388 total) in human tumor array data that were corresponding to PTEN-regulated probes were reduced by a two-sample *t* test: adjusted $P > .05$ (no statistical difference) between the benign and localized samples and the adjusted $P < .05$ (statistically different) between the localized and metastatic samples. The resulting 61 up- (C) and 21 down-regulated (D) PTEN-responsive probe sets were clustered with human tumor array data, and the expression patterns of these probe sets among human prostate tumor samples (benign, localized, and metastatic) were shown with corresponding heat maps.

Lipid Phosphatase Activity Is Required for PTEN Inhibition of Cdc6 and Cyclin E2 Expression

PTEN has both lipid and protein phosphatase activity, and its lipid phosphatase activity has been associated with its growth inhibitory effect. Because inhibition of cdc6 and cyclin E2 by PTEN was implicated in PTEN cell cycle G_1 arrest, we sought to determine whether its lipid phosphatase activity is involved in its inhibition of *cdc6* and *cyclin E2* expression levels by examining expression levels of these two genes after the induction of G129E mutant PTEN that lacked lipid phosphatase activity in C4-2 (Figure 6*A*) [11]. In contrast to wild type PTEN, expression of G129E mutant PTEN had no effect on *cdc6* and *cyclin E2* expression levels (Figure 6, *B* and *C*). Consistently, no significant change of cell cycle distribution was observed after G129E mutant PTEN expression (Figure 6*D*), and the minimal alteration of cell cycle was probably caused by the effect of DOX itself [11]. Inhibition of Akt (Figure 6*E*), the major downstream target of PTEN lipid phosphatase activity, by an Akt-specific inhibitor, Akt inhibitor VIII [22], significantly decreased expression levels of *cdc6* and *cyclin E2* (Figure 6*F*). In contrast, inhibition of the JNK signaling pathway (identified as a PTEN functional target in parallel to Akt [23]; Figure 6*E*) with a specific JNK inhibitor, SP600125 [24], showed no effect on the expression of these two genes. Collectively, these results suggest that the inhibition of cdc6 and cyclin E2 expression by PTEN is dependent on its lipid phosphatase activity and is mediated through the Akt pathway.

PTEN Regulates Cdc6 Expression through the E2F Transcription Factor in C4-2 Cells

Similar changes in the magnitude of mRNA and protein expression of cdc6 and cyclin E2 in response to PTEN induction (Figure 3, *B* and *C*) suggest that these are transcriptionally regulated. Because expression levels of cdc6 and cyclin E2 can be regulated by the E2F transcription factor [19,25], here we evaluate whether PTEN exerts its effect on cdc6 and cyclin E2 through E2F. We examined whether PTEN could affect E2F transcriptional regulation by examining E2F reporter activity after PTEN expression. E2F luciferase-reporter construct was transiently transfected into the C4-2 cells, and the expression of PTEN inhibited E2F reporter activity approximately 40% (Figure 7*A*), a similar decrease to that seen in mRNA and protein expression of cdc6 and cyclin E2, and this inhibition of E2F reporter activity was not caused by the effect of PTEN on E2F expression levels (Figure 7*A*). Surprisingly, PTEN expression had no effect on the phosphorylation levels of Rb, a known factor that regulates E2F transcriptional activity. To determine the role of E2F in cdc6 transcriptional regulation by PTEN, we transiently transfected wild type and mutant

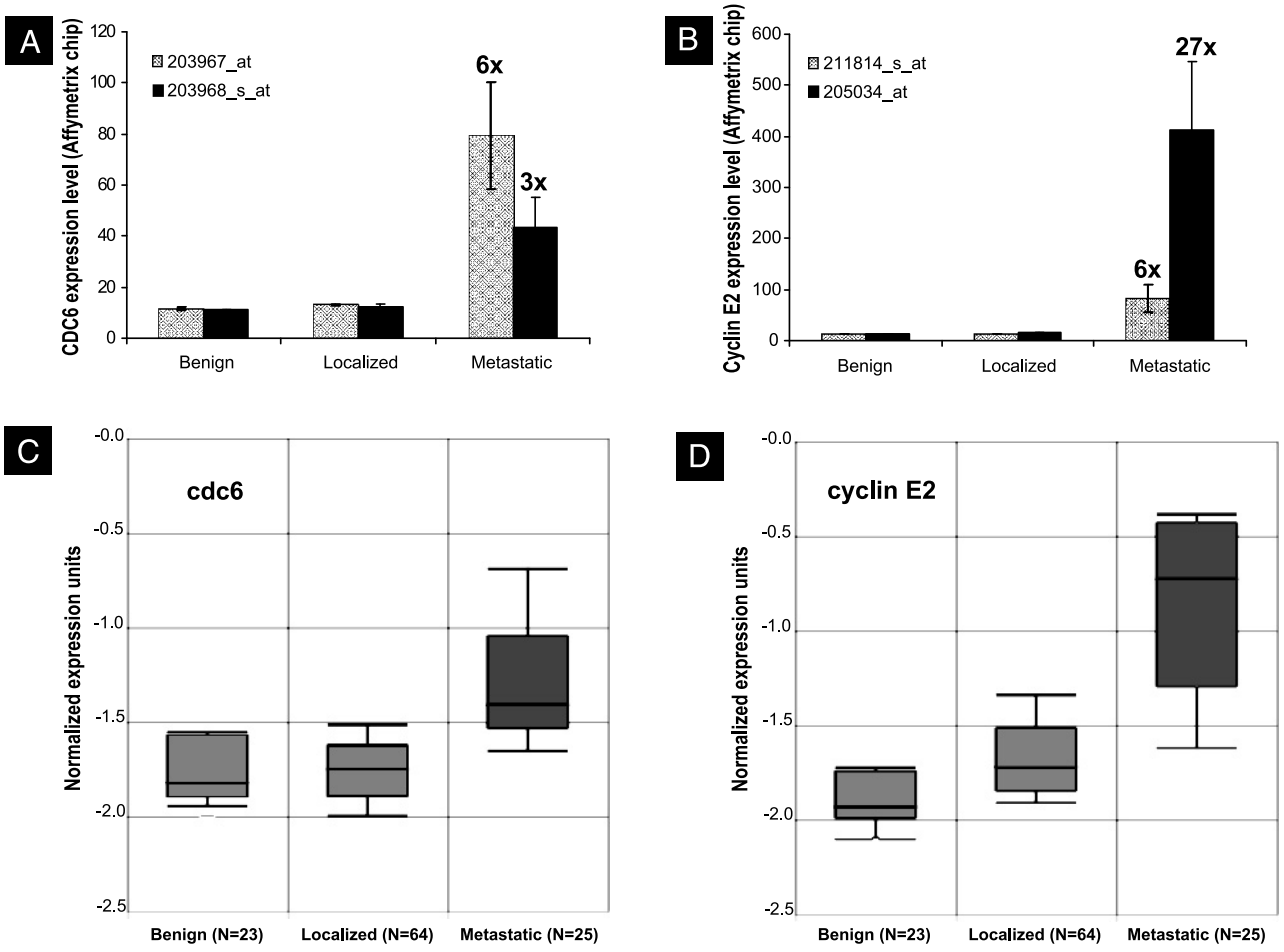

Figure 3. Cdc6 and cyclin E2 expression levels in human prostate cancer tumors. Cdc6 (A and C) and cyclin E2 (B and D) expression levels in two independent human tumor array data sets. All probe sets available for each gene are shown. Data in panels A and B were derived from study conducted by Varambally et al. [15], and data in C and D were obtained from an Oncomine study [20]. Numbers on top of bar graph in A and B show fold difference compared to localized samples. Error bars, SEM.

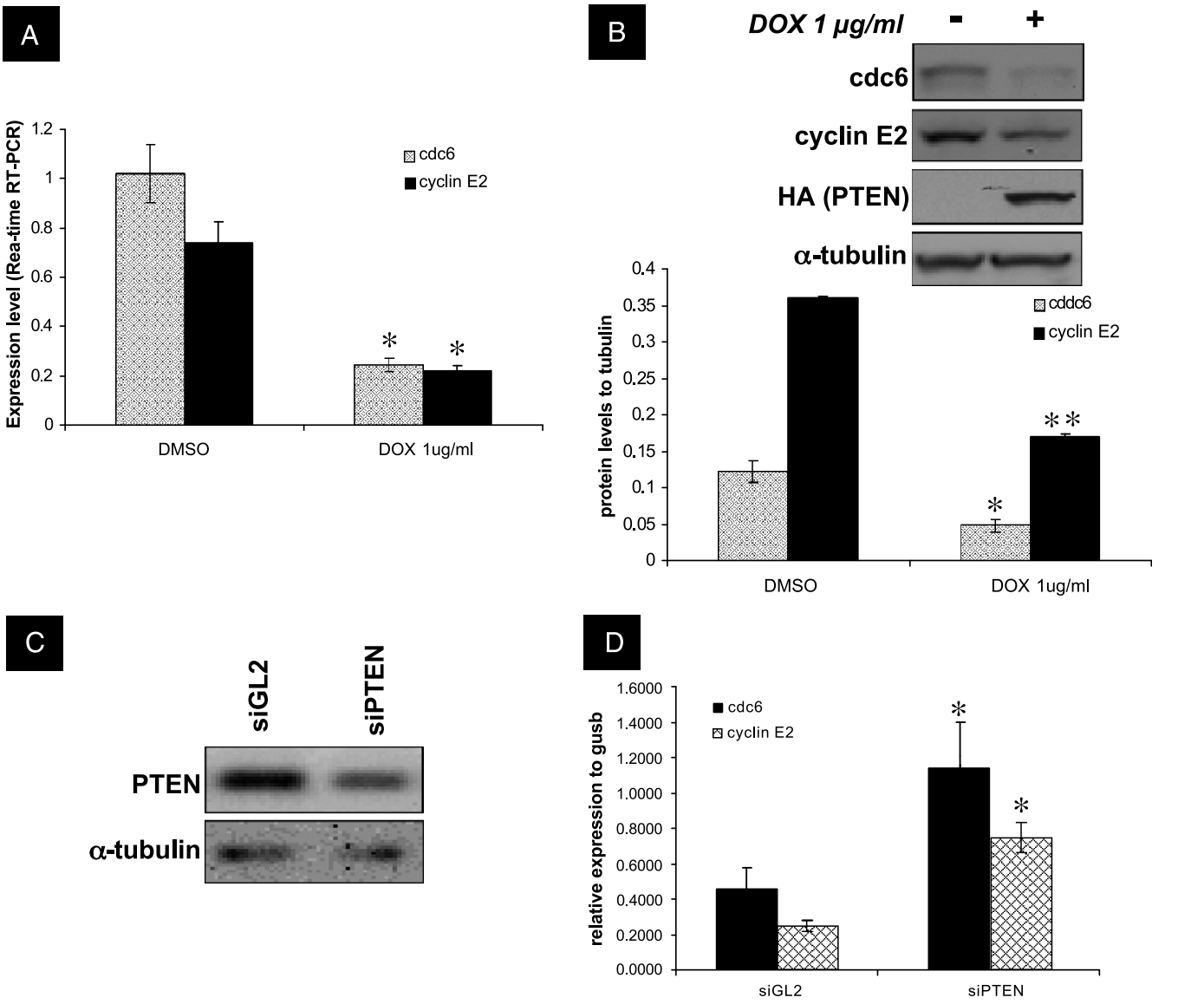

Figure 4. Expression levels of cdc6 and cyclin E2 in response to PTEN. mRNA (A) and protein (B) expression levels of cdc6 and cyclin E2 after PTEN expression. pTetOn PTEN C4-2 cells were treated with 1 μg/ml DOX for 24 hours to reexpress PTEN; total RNA and cell lysate were extracted from duplicate samples to do real-time RT-PCR and Western blot, respectively, to determine mRNA and protein expression levels of cdc6 and cyclin E2. Graph represents densitometry analysis of cdc6 and cyclin E2 protein levels from two independent experiments after PTEN expression with 1 μg/ml DOX treatment for 24 hours. Protein levels of cdc6 and cyclin E2 were normalized by levels of α-tubulin (inset). (C) Western blots showing siRNA-mediated knockdown of PTEN (siPTEN) in prostate cancer DU145 cells after 72 hours of transient transfection of siRNA. (D) Corresponding *cdc6* and *cyclin E2* mRNA expression levels after siRNA-mediated knockdown of PTEN, in DU145 cells were determined with real-time RT-PCR. *$P < .05$ and **$P < .01$, respectively, compared to DMSO treatment or control siRNA targeting luciferase (siGL2).

cdc6 promoter (mutations in both E2F binding sites) constructs into C4-2 cells and examined cdc6 promoter activity after PTEN expression. Whereas wild type cdc6 promoter activity was decreased approximately 35% after PTEN expression, the activity of mutant cdc6 promoter was unaffected by PTEN expression (Figure 7*B*). These results suggest that PTEN expression inhibits expression of cdc6 through the E2F transcription factor.

The effect of PTEN on E2F transcriptional activity prompted us to examine whether PTEN modulates other E2F-regulated genes and whether those genes are also associated with human prostate cancer metastasis. We used a computational tool called PAINT [17] to uncover putative transcription factor–regulatory pathways associated with gene down-regulation by PTEN. By looking at overrepresented transcription factor binding sites in the regulatory regions of PTEN down-regulated genes, we found that E2F was among the most significant predications by PAINT of transcription factors regulated by PTEN (Table 2). In the clustering analysis (Figure 7*C*), PAINT identified two additional PTEN-regulated E2F genes, denticleless homolog (*DTL*) and DNA fragmentation factor (*DFFB*). We then evaluated their expression levels in human prostate cancer [15]. Figure 7*D* indicates that levels of *DFFB* did not change, whereas expression levels of *DTL* were increased dramatically in metastatic samples compared to benign and localized samples. These results further support the regulatory role of E2F transcription activity by PTEN

and indicate an association of PTEN-regulated E2F genes and human prostate cancer metastasis.

Discussion

In prostate cancer, loss of PTEN has been associated with more advanced disease and previous studies indicate that PTEN regulates prostate cancer androgen sensitivity and bone tropism [11,26]. Using a PTEN-deficient human prostate cancer cell line harboring an inducible PTEN expression construct, we defined a PTEN gene expression signature. We then evaluated the association of this signature with tumor stage from profiling data on human prostate cancers. Finally, by filtering genes which are both suppressed by PTEN and elevated as a function of human tumor metastasis, we defined candidate biomarkers or effectors of PTEN-mediated metastasis associated pathways. We found that genes regulated by PTEN expression could clearly distinguish metastatic prostate tumor samples from benign and localized prostate tumor tissues, supporting the idea that PTEN-associated transcriptional programs are involved in the late stages of tumor progression in prostate cancer. Although we cannot rule out possible secondary effects of PTEN expression, the relatively short period of PTEN induction suggested a more direct role of PTEN in transcriptional regulation of those genes.

Our approach is complementary to that used in two recent reports examining the link between transcriptional signatures associated with PTEN loss and cancer prognosis [10,27]. In contrast to our approach, these investigators first used patient material or human xenografts with characterized PTEN status to develop a gene signature. The nine-gene signature discovered by Mehrian-Shai et al. reflected PTEN status in a set of human glioblastoma and human prostate cancer xenografts. Of these nine genes, the secreted protein IGFBP-2 was found to be a potential biomarker of PTEN status and might possibly serve as a tool to monitor PI3K signaling in pathway-specific targeted treatment. Similarly, stathmin was found as a surrogate marker for the 246–PTEN gene signature discovered from human breast cancer samples, and those signature genes not only reflected PTEN-associated PI3K signaling pathway alternation but also integrated various PI3K pathway lesions. In addition, the 246-gene signature was associated with a significantly worse distant disease-free survival in both breast and prostate cancer tumor samples. Interestingly, none of the signature genes of Mehrian-Shai et al. were present among our PTEN-regulated genes, and only one common gene was found between our data set and that of Saal et al., *PTEN* itself. Similarly, there was overlap of only one gene, *TUA8 Cri-du-chat* region, between Mehrian-Shai et al. and Saal et al. The lack of overlap among signatures that predict disease stage or recurrence is not unusual [28] and is likely caused by the different approaches used by our study and the other two studies. Despite this, such signatures can be robustly predictive of disease outcome [29].

The role of PTEN and related PI3K-Akt pathway in transcriptional regulation has been explored in numerous gene expression microarray studies [30,31], and it would seem that multiple mechanisms are involved. Our study indicates that regulation of cdc6 and cyclin E2 expression by PTEN is mediated through its effect on PI3K-Akt signaling because the G129E mutant PTEN has no inhibition on their expression. Expression of cyclin E2 has been related to PTEN status

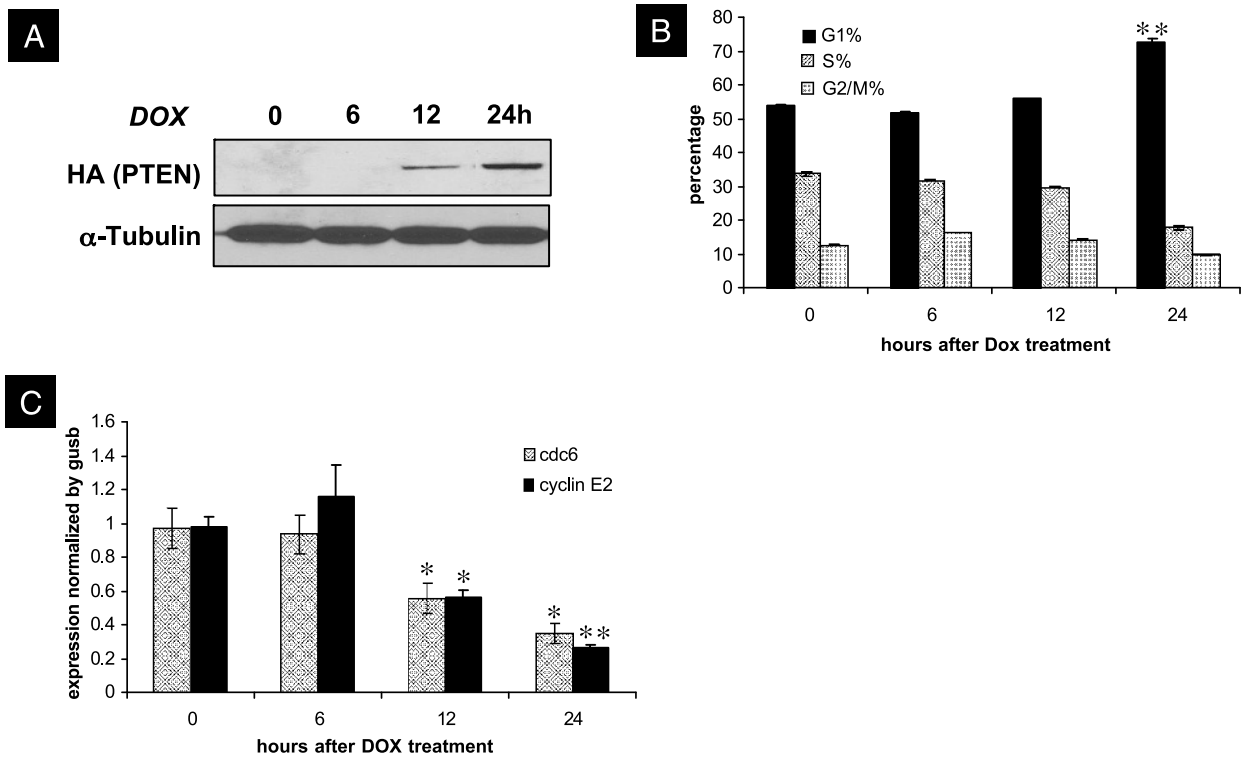

Figure 5. Time course study of PTEN, cdc6, and cyclin E2 expression and cell cycle distribution. pTetOn PTEN C4-2 cells were treated with DOX for 6, 12, and 24 hours to induce PTEN expression. (A) The expression of PTEN was determined by Western blot with an anti-HA antibody. (B) Correlated cell cycle distribution was determined by propidium iodide staining as described in Materials and Methods with a duplicate sample. (C) Total RNA from a triplicate sample was isolated to examine *cdc6* and *cyclin E2* mRNA levels by real-time RT-PCR. Error bars, SEM. **P* < .05 and ***P* < .01, respectively, compared to 0 time point.

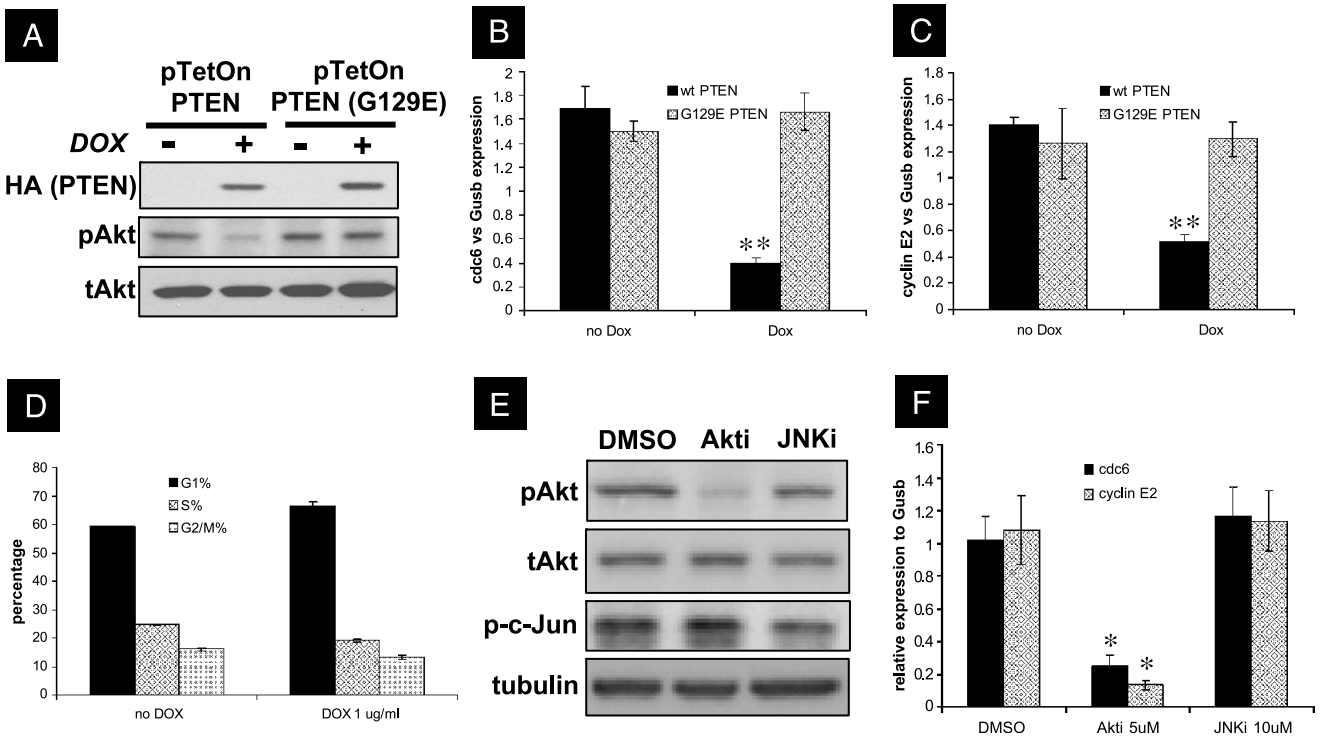

Figure 6. Role of PTEN lipid phosphatase activity in cdc6 and cyclin E2 expression. (A) Western blot characterization of wild type and G129E mutant PTEN reexpression in pTetOn PTEN and pTetOn PTEN (G129E) C4-2 cells after 1 μg/ml DOX treatment for 24 hours. Cdc6 (B) and cyclin E2 (C) expression levels were determined by real-time RT-PCR after wild type (wt) or G129E mutant PTEN expression. (D) Correlated cell cycle distribution was determined by propidium iodide staining as described in Materials and Methods with a duplicate sample. (E) Western blots showing inhibition of phospho-Akt (pAkt) with 5 μM Akt inhibitor (Akti), Akt inhibitor VIII, and decreased phospho-c-Jun levels with 10 μM JNK inhibitor (JNKi), SP600125, in pTetOnPTEN C4-2 cells after 24 hours of treatment. (F) Total RNA in C4-2 cells treated with Akt and JNK inhibitors in (E) was extracted to determine mRNA levels of *cdc6* and *cyclin E2* by real-time RT-PCR. Error bars, SEM. $^*P < .05$ and $^{**}P < .01$, respectively, compared to control (no DOX or DMSO treatment).

because elevated cyclin E2 levels were observed in epidermal sites after conditional PTEN ablation [32]. Our work demonstrates a causal linkage and further indicates that the regulation of cdc6 expression by PTEN is mediated through the E2F transcriptional factor. E2F is involved in the regulation of multiple genes that are essential to cell cycle G_1-to-S transition including cdc6 and cyclin E2 [19,25], and our results suggest that PTEN could affect E2F transcription activity. E2F activity is inhibited on binding to hypophosphorylated Rb [33] whose phosphorylation is inhibited by PTEN, and this inhibition is rescued by the expression of a catalytically active subunit of PI3K [34]. However, PTEN expression did not change phosphorylation levels of Rb in our C4-2 cells suggesting that Rb was not involved in PTEN inhibition of E2F activity here. Alternatively, PTEN might directly affect E2F function because Shen et al. [35] reported that PTEN interacted with E2F to influence the transcriptional regulation of Rad51. Recently, inhibition of the PI3K-Akt downstream target, GSK-3β, by either inhibitor or siRNA treatment decreased cdc6 and cyclin E2 expression, and this inhibition was suggested to be mediated through E2F in prostate cancer cells [36]. Together with our results, these studies suggest potential roles of PI3K-Akt and E2F in the regulation of cdc6 and cyclin E2 expression by PTEN.

The cell cycle G_1 arrest by PTEN has been reported previously [37], and we observed similar result in C4-2 cells [11]. Cdc6 is involved in cell cycle G_1-to-S transition and siRNA knockdown of cdc6 expression caused cancer cell G_1 arrest and apoptosis because it is essential to DNA replication [38]. Similarly, cyclin E2 has been associated with cell cycle G_1- to S-phase transition by associating and activating of CDK2, and its overexpression decreased cell cycle G_1 length [21]. Our studies here show a significant decrease of *cdc6* and *cyclin E2* expression before the cell cycle G_1 arrest, suggesting that the inhibition of these two genes by PTEN is not simply caused by the global inhibition of PTEN cell cycle G_1 arrest. Rather, the inhibition of *cdc6* and *cyclin E2* by PTEN could be involved in its cell cycle G_1 arrest function given the essential roles of these two genes in cell cycle G_1- to S-phase transition.

It should be noted that the cell cycle arrest in C4-2 cells after PTEN restoration could potentially skew our PTEN-regulated metastasis-associated gene lists toward cell cycle–regulatory genes that impact metastasis by regulating growth at the distant site. Thus, other gene families that regulate metastasis may not have been identified as easily if overshadowed by these growth-related genes. Further direct evidence would be needed to determine directly whether these cell cycle–regulatory genes are actually involved in the metastatic colonization process [39]. Nevertheless, there is significant evidence that *cdc6* and *cyclin E2* are associated with progression in human cancer. For example, enhanced expression of *cdc6* was found in metastatic melanomas, and increased expression in primary melanomas was associated with bad prognosis [40]. The expression level of *cyclin E2* was increased and associated with decreased metastasis-free survival and overall survival in breast cancer [41]. Our results implicating increased expression of *cdc6* and *cyclin E2* in human metastatic prostate cancer tissues in two microarray studies from independent centers [15,20] are consistent

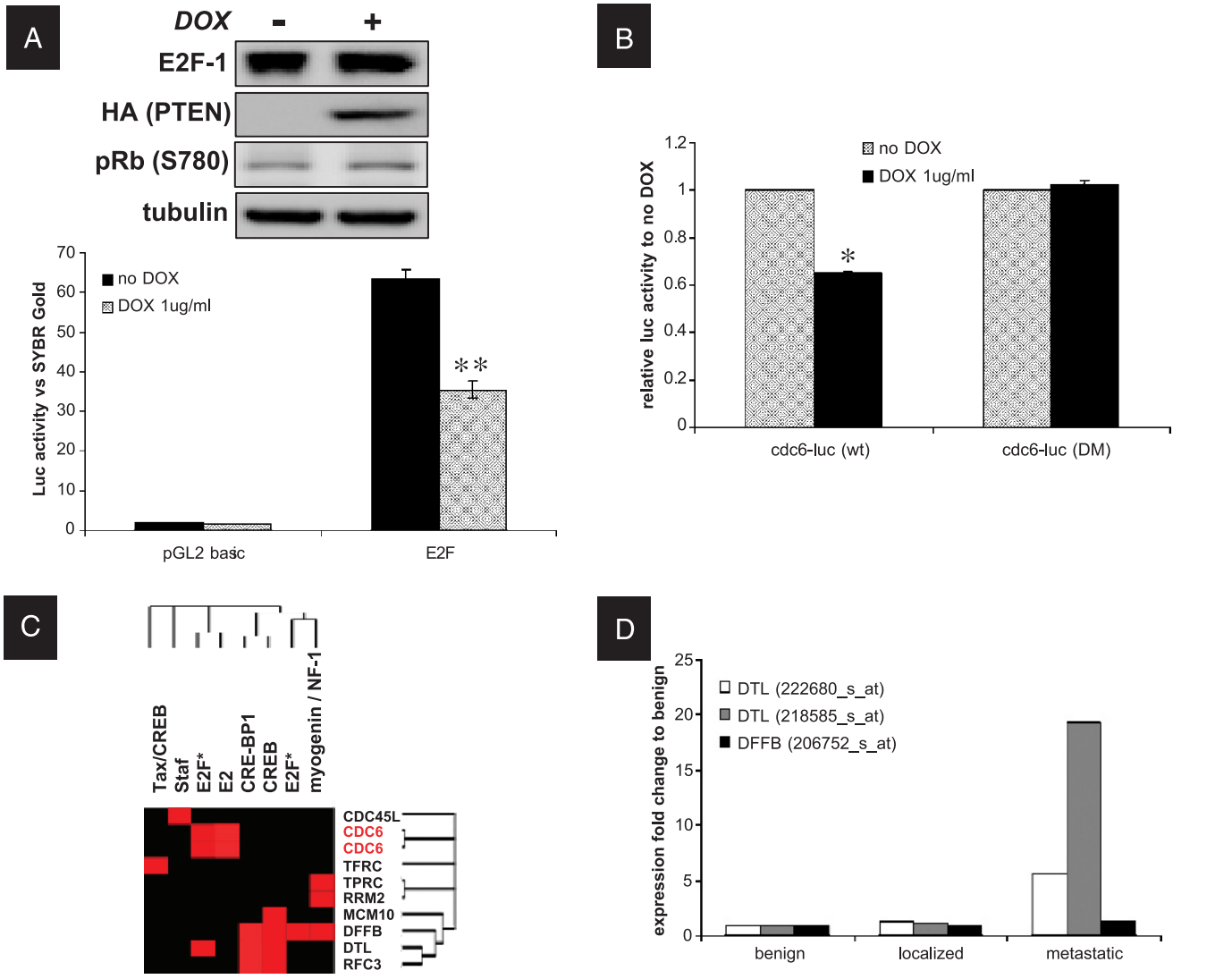

Figure 7. Effects of PTEN expression on E2F transcriptional activity and cdc6 promoter activity. (A) E2F reporter luciferase construct was transiently transfected into pTetOn PTEN C4-2 cells. Cells were treated with 1 μg/ml DOX for 24 hours, and E2F reporter activity was measured by luciferase assay. Inset: Western blots showing expression levels of E2F-1 and phosphor-Rb (S780) after PTEN expression. (B) Wild type (wt) or mutant (DM, double mutation on both E2F binding sites) cdc6 promoter-luciferase construct was transiently transfected into pTetOn PTEN C4-2 cells. Cdc6 promoter activity was determined by luciferase assay after PTEN expression in cells treated with 1 μg/ml DOX for 24 hours. The luciferase activity of DOX treatment was normalized to no DOX in both wild type and mutant groups. (C) PAINT clustering analysis showing interaction matrix for statistically overrepresented transcriptional regulatory elements (TREs) in PTEN down-regulated genes. The corresponding PTEN down-regulated genes whose upstream sequences harbor those TREs were listed in the column next to the matrix. (D) Relative expression levels of probe sets representing DTL and DFFB in human prostate tumor array data [15]. All probe sets available for each gene were shown. Data indicate fold change difference compared to benign. Error bars, SEM. *$P < .05$ and **$P < .01$, respectively, compared to no DOX treatment.

Table 2. List of Significantly Overrepresented ($P < .05$) Transcription Factors in PTEN Down-regulated Genes.

Transcription Factor	$P^{\dagger}$
E2	.0004
CRE	.0051
E2F*	.0119
Staf	.0243
CREB	.0248
Tax/CREB	.0315
myogenin/NF-1	.0433
E2F*	.0465

$^{\dagger}P$ values were calculated by PAINT Web service (http://www.dbi.tju.edu/dbi/tools/paint/).
*Multiple listings of same transcriptional factors are based on differences in consensus binding sites.

with these studies. Future IHC studies in metastatic prostate cancer tissues need to be carried out to determine whether protein levels of *cdc6* and *cyclin E2* are correlated to their mRNA levels in metastatic samples. Given our data, such correlation is likely.

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Competing interests statement

The authors declare that they have no competing financial interests.

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