Valproic acid inhibits the proliferation of cancer cells by re-expressing cyclin D2

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In this study, primary murine prostate cancer (PCa) cells were derived using the well-established TRAMP model. These PCa cells were treated with the histone deacetylase inhibitor, valproic acid (VPA), and we demonstrated that VPA treatment has an antimigrative, antiinvasive and antiangiogenic effect on PCa cells. Using microarray analyses, we discovered several candidate genes that could contribute to the cellular effects we observed. In this study, we could demonstrate that VPA treatment of PCa cells causes the re-expression of cyclin D2, a known regulator that is frequently lost in PCa as we could show using immuno-histochemical analyses on PCa specimens. We demonstrate that VPA specifically induces the re-expression of cyclin D2, one of the highly conserved D-type cyclin family members, in several cancer cell lines with weak or no cyclin D2 expression. Interestingly, VPA treatment had no effect in fibroblasts, which typically have high basal levels of cyclin D2 expression. The re-expression of cyclin D2 observed in PCa cells is activated by increased histone acetylation in the promoter region of the Ccnd2 gene and represents one underlying molecular mechanism of VPA treatment that inhibits the proliferation of cancer cells. Altogether, our results confirm that VPA is an anticancer therapeutic drug for the treatment of tumors with epigenetically repressed cyclin D2 expression.

Introduction

In the USA, prostate cancer (PCa) is the second most commonly diagnosed cancer in men and is the leading cause of cancer-related deaths in men (1). There are very few treatment options for PCa: radiation therapy, androgen ablation therapy and radical prostatectomy are the main opportunities. However, all of these treatments can severely impair the patient’s quality of life (2). The characterization of cancer cells is an important step toward the development of new therapeutic strategies to treat this disease. Cancer research was and is done mainly on the genetic level, but accumulating results show that also changes in epigenetic patterns of cells are crucial for tumor onset and progression (e.g. histone acetylation, histone methylation and DNA methylation) (3).

Acetylation is one of eight different ways to covalently modify histone proteins; it causes a neutralization of formerly positively charged lysine residues (4,5). Subsequently, acetylation loosens the chromatin structure, and the chromatin becomes transcriptionally active (4). The process of acetylation/deacetylation is catalyzed by histone acetyltransferases and histone deacetylases, respectively. Deregulating the balance between histone acetyltransferases and histone deacetylases leads to aberrant transcription and to the development of cancer (5).

Histone deacetylase inhibitors (HDACis), such as valproic acid (VPA), inhibit histone deacetylases causing increased chromatin acetylation (6). VPA as a potentially useful drug to treat PCa benefits from its long experience as an anticonvulsant; it is already used in several clinical trials, for example, against high-risk acute myeloid leukemia, refractory solid or central nervous system tumors and breast and cervical cancers (7–9).

PCa cells, as with many other cancer cells, are characterized by an increased ability to proliferate, migrate and invade surrounding tissues (10). Recent studies demonstrate that treating PCa cells with VPA, diminishes cell proliferation and migration (11–16). However, the molecular mechanisms behind these observations are not yet fully elucidated and, remarkably, these effects are cell type specific (17).

Concerning the inhibition of cancer cell proliferation using VPA, one major field for investigations of mechanistic functions of VPA is the regulation of cell cycle genes. D-type cyclins are an important group of highly conserved cell cycle regulators. Generally, the family member cyclin D2 is a key player in cell cycle progression from the G1 phase to S phase (18). However, in cancer development, cyclin D2 plays different roles depending on the tumor type. In certain tumors, cyclin D2 is overexpressed, whereas in patients with PCa or mammary cancers, the promoter region of cyclin D2 is hypermethylated, which causes a loss in cyclin D2 expression (19–23). Interestingly, in the PCa cell line LNCaP, restoring cyclin D2 expression inhibits cell proliferation (24) and in non-small cell lung cancer, the reduced expression of cyclin D2 is correlated with a poor recurrence-free survival (25). These latter results led to our hypothesis that cyclin D2 functions in some cancers, such as PCa, as a tumor suppressor rather than an oncogene. Thus, cyclin D2 could serve as a putative candidate target for PCa treatments (26).

In this work, we detected a decrease in the invasiveness, migration and proliferation of murine primary PCa cells after VPA treatment. To investigate the underlying causes of these cellular changes following VPA treatment, we performed gene expression profiling. One of the candidate genes we identified was cyclin D2, which is specifically re-expressed after VPA treatment by increase of the acetylation of the Ccnd2 promoter. In various cancer cells with a low basal expression of cyclin D2, VPA treatment caused re-expression, accompanied by an inhibition of proliferation.

Materials and methods

Cell culture

PC-3, DU145, LNCaP, DLD-1, SW620 and SW480 cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS) and 1.2% penicillin/streptomycin (all PAN-Biotech GmbH, Aidenbach, Germany). HEK293, L cells and NIH/3T3 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% FCS, and Caco-2 and MRC-5 cells were grown in modified Eagle’s medium with 20% FCS; both contained 1% penicillin/streptomycin (MSC-5 was a kind gift from M.Rave-Fränk, Department of Radiotherapy and Radiooncology, University Medical Center, Göttingen, Germany; both media were from Life Technologies GmbH, Darmstadt, Germany). Mouse 2E PCa cells were generated using a C57/BL6 TRAMP tumor and cultured, as described by Kaulfuß et al. (27). NXS1 and Wt fibroblasts were a kind gift from E.Nitzi (Department of Developmental Genetics, University Medical Center, Göttingen, Germany) and were established from the dermis of Pten+/−/+ and wild-type C57/BL6 mice, respectively, according to the protocol described by Uhrmann et al. (28). The prostate stromal cells were obtained from Lonza (Basel, Switzerland) and maintained according to the manufacturer’s instructions.

Abbreviations: cDNA, complementary DNA; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; FCS, fetal calf serum; HDACi, histone deacetylase inhibitors; NaB, sodium butyrate; PCa, prostate cancer; qRT–PCR, quantitative reverse transcription–PCR; SAHA, suberoylanilide hydroxamic acid; siRNAs, small interfering RNAs; TRAMP, transgenic adenocarcinoma of mouse prostate; TSA, trichostatin A; VPM, valpromide; VPA, valproic acid.

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Western blot analyses
Western blot analyses were performed, as described by Kaulfuss et al. (29); the proteins were lysed using a lysis buffer, as described previously (30). The signals were captured using a FluoroChem Q (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and analyzed using the FluoroChem Q SA Software (Biozym Scientific GmbH). The following are the primary antibodies used for the western blot analyses: Ac-Histone H3 (Lys 2) (sc-34262; Santa Cruz Biotechnology, Heidelberg, Germany), cyclin D2 (D252P9) Rabbit Monoclonal Antibody #53741, cyclin D3 (DCS22) Mouse Monoclonal Antibody #29356 (both Cell Signaling Technology, Danvers, MA) and SV40 large T Antigen (554149, Pab101; BD Pharmingen™, Heidelberg, Germany). The following secondary antibodies were used: peroxidase-conjugated AfiniPure rabbit anti-mouse IgG and goat antirabbit IgG (Dianova, Hamburg, Germany).

Chemicals
The following chemicals were used: VPA (Sigma-Aldrich, St Louis, MO), valproamide (VPM; Alfa Aesar, Karlsruhe, Germany), sodium butyrate (NaB; Alfa Aesar), trichostatin A (TSA), apicidin (both AppliChem GmbH, Darmstadt, Germany) and suberoylanilide hydroxamic acid (SAHA; Cayman Chemical Company, Ann Arbor, MI).

Real-time reverse transcription–PCR analysis
For reverse transcription, the total RNA was harvested using thepeqGOLD Total RNA kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and transcribed to complementary DNA (cDNA) using SuperScript® II Reverse Transcriptase (Life Technologies GmbH) and an Oligo-deoxynucleotide primer (Eurofins MWG Operon, Ebersberg, Germany). For real-time PCR, Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Life Technologies GmbH) was used; the reaction and data collection were performed using the ABI Prism 7900T Sequence Detection System (Life Technologies GmbH). The program for the PCR consisted of the following cycles: 50°C for 2 min, 95°C for 3 min, 40 cycles (94°C for 15 s, 60°C for 30 s, 72°C for 30 s, 95°C for 15 s, 60°C for 15 s and 95°C for 15 s). The messenger RNA expressions of the housekeeping genes (Hprt and Tbp) were used for normalization of the human samples, and the Hprt (hypoxanthine–guanine phosphoribosyltransferase) and Tbp for the mouse samples. The relative expression was calculated using the ΔΔCT method. The primers used for quantitative reverse transcription–PCR (qRT–PCR) are specified in the Supplements.

Immunocytochemistry
Cells were cultured for 24 h on four-well chamber culture slides (BD Pharmingen™). Cells were fixed, treated and mounted, as described by Kaulfuss et al. (29). The following antibodies were used: SV40 clone 101 (BD Pharmingen™), sheep antimonucye-Cy3 (Sigma–Aldrich) and fluorescein isothiocyanate-conjugated phallolidin (Sigma–Aldrich), which was used to stain F-actin. The images were captured using a confocal laser scanning microscope FluoView1000 (Olympus, Hamburg, Germany).

Proliferation assay
The proliferation assays were performed according to the manufacturer’s instructions using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Mannheim, Germany). Using 96-well plates, 3000 cells per well were plated and incubated in the presence or absence of VPA; cell proliferation was measured after 72 and 144 h. At least three independent experiments were performed in triplicate.

 Colony forming assay
The 2E PCa cells were seeded at a low density (800 cells per well of a six-well plate) and, after 24 h of incubation, the cells were treated with varying concentrations of VPA. The medium was routinely changed twice a week. When the control cells formed clear colonies (approximately 14 days), the cells were fixed in ethanol for 20 min and subsequently stained with hematoxylin/eosin. The colonies were counted using a light table, and three independent experiments were performed.

Migration and invasion assay
For the in vitro cell migration assays, 2E PCa cells were pre-treated with VPA for 48 h. Subsequently, 70,000 cells were transferred into the Millicell 8.0 µm hanging PET inserts (Millipore, Billerica, MA) and were incubated for 24 h under normal culture conditions. The in vitro cell invasion from 2E cells was determined using BioCoat Matrigel Invasion Chambers (BD Pharmingen™). Here, cells were pre-treated with VPA for 24 h, counted and 70,000 cells were plated in medium containing 10% FCS into the inserts. The medium in the culture plates contained 20% FCS to create a gradient. The cells were incubated for 48 h, fixed, stained and counted, as described previously (29).

Microarray analysis
Three biological replicates were analyzed according to the method described by Meyer et al. (31). Briefly, after checking the RNA integrity, double-stranded cDNA was synthesized and double-stranded cDNA cleanup was performed according to the manufacturer’s instructions (Affymetrix, Santa Clara, CA). Using the WT Target Labeling kit (Affymetrix), in vitro transcription and single-stranded DNA synthesis was performed. After enzymatic protection and labeling, biotinylated single-stranded DNA was hybridized onto the GeneChip® Mouse Gene 1.0 ST Array (Affymetrix) according to the manufacturer’s instructions. After antibody signal amplification, washing and staining with streptavidin-R phycoerythrin (Life Technologies GmbH), the arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix). The generated data conform to the Minimum Information About a Microarray Experiment (MIAME) guidelines, have been deposited in NCBI’s Gene Expression Omnibus and are accessible through the GEO Series, accession number GSE34072 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34072).

siRNA transfection
The transfection of NIH/3T3 fibroblasts was performed using TurboFect™ siRNA Transfection Reagent (Fermentas, St Leon-Rot, Germany) according to the manufacturer’s instructions and using three independent, specific small interfering RNAs (siRNAs) against cyclin D2 (MSS236126, MSS236127, MSS236128; Stealth RNAi™ siRNA, Life Technologies GmbH) at a concentration of 50 nM in the transfection media. The control cells were transfected with siRNA duplex oligonucleotides against the firefly luciferase gene (Eurogentec, Cologne, Germany). Twenty-four hours after transfection, the cells were transferred to the proliferation assay. The effect of the cyclin D2 knockdown in the NIH/3T3 cells was tested using western blot analysis 24 h after the siRNA transfection.

Chromatin immunoprecipitation
For DNA shearing, the Shearing Chip kit (Diagenode, Liège, Belgium) was used according to the manufacturer’s instructions, and samples were sheared using the Bioruptor UCD-200 (Diagenode) set on ‘high’ for 30 min with 30 s ON and 30 s OFF. The crushed ice in the water bath was changed every 10 min to keep the samples cool. Immunoprecipitation was performed using the OneDay Chip kit (Diagenode) according to the manufacturer’s protocol and the specific CHIP-ACtetyl-Histone H3 antibody (Lys 9) (17–658; Millipore) overnight. For the qPCR analyses, the immunoprecipitated DNA was diluted 1:2, and the analyses of results were performed using the % input method with a subsequent normalization to input. (The primer sequences for the qRT–PCR after the chromatin immunoprecipitation [ChIP] are available on request.)

Immunohistochemistry
Tumor-free (n = 6) and tumor prostate tissues (n = 15) as well as one lymph node metastasis were acquired from 21 male patients from the University Medical Centre in Gütingen, Germany. The study was approved by the ethics committee. Immunohistochemical reactions were performed on 3–5 µm formaldehyde-fixed and paraffin-embedded tissue sections. After deparaffinization and rehydration, the sections were incubated in citrate buffer (pH 6) for antigen retrieval. The sections were incubated for 30 min at room temperature with polyclonal antibodies against cyclin D1 at a 1:100 dilution (NeoMarkers, Fremont, CA) and cyclin D2 at a 1:200 dilution (Santa Cruz Biotechnology). Thereafter, biotinylated secondary antibody (REAL biotinylated secondary antibodies; Dako, Hamburg, Germany) and streptavidin-alkaline phosphatase were applied; Fast red (Dako) was used as the chromogen. The tissue samples were analyzed using light microscopy after counterstaining with Meyer’s hematoxylin.

Results
Treatment with VPA induces histone acetylation in a new murine primary PCa cell line and in an established human PCa cell line
To generate an in vitro cell model for PCa that is adaptable to the transgenic adenocarcinoma of mouse prostate (TRAMP) model, we established a murine primary PCa cell line from a TRAMP tumor, as described previously (27,32), which was named 2E after its derivation. Due to the introduced promoter of rat probasin in front of the SV40 T/t antigen only epithelial cells of mouse prostate express the transgene. Thus, to prove the epithelial origin of the 2E PCa cells, immunofluorescence analysis was performed using a specific antibody against the SV40 large T antigen. We demonstrated that 100% of the 2E cells examined were positive for expression of the
SV40 large T antigen (Supplement 1A, available at Carcinogenesis Online). The strong expression of SV40 large T antigen in 2E PCa cells was also detected by western blot analysis (Supplement 1B, available at Carcinogenesis Online).

Using the aforementioned in vitro model, 2E PCa cells, the histone deacetylase activity of VPA was tested. The western blot analyses demonstrated that the acetylation of histone 3 at lysine 9 was increased in a dose-dependent manner (Supplement 1C, available at Carcinogenesis Online), and similar results were obtained using the established human PCa cell line PC-3 (Supplement 1C, available at Carcinogenesis Online).

On the cellular level, VPA inhibits the migration, invasion and proliferation of the new primary 2E PCa cells

To determine the functional effects of VPA on the 2E PCa cells, both cell migration and invasion were analyzed. The murine primary 2E PCa cells showed a decreased migratory ability after 72h of VPA treatment in a dose-dependent manner, ranging from 28% for the low VPA concentration (5 mM) up to 51% for the high VPA concentration (10 mM) in comparison with the control cells (Figure 1A). The treatment of 2E PCa cells with 5 mM VPA for 72h reduced their invasiveness up to 52% compared with the control-treated cells (Figure 1B). However, treating with a higher VPA dose (10 mM) did not further inhibit their invasiveness.

With regard to proliferation of 2E cells, VPA treatment caused a clear dose- and time-dependent reduction (Figure 1C). Their proliferative activity was diminished after 72h of VPA treatment (data not shown). After 144h, treatments using a low concentration of VPA (1 mM) decreased the proliferation rate to 68%, and treatments using a high concentration of VPA (10 mM) decreased the proliferation rate to 28%, compared with the control-treated cells (Figure 1C). In addition, the PC-3 cells demonstrated a decreased proliferation rate after VPA treatment, in a dose- and time-dependent manner (Supplement 2, available at Carcinogenesis Online). Finally, a colony formation assay was performed on the murine 2E PCa cells, and as depicted in Figure 1D, high VPA concentrations strongly inhibited 2E cell colony formation.

Several genes are differentially expressed in the new primary 2E PCa cell line after VPA treatment

To analyze the molecular changes in the primary 2E PCa cells upon VPA treatment, gene expression microarray analysis was performed. The 2E cells were treated with 1 mM VPA for 24h to identify the VPA-sensitive genes that are directly regulated by VPA-mediated acetylation and to exclude any secondary effects caused by deregulated acetylation-sensitive gene products. The raw data were normalized and filtered, as described in the Materials and methods. To further increase the biological relevance of the differentially expressed genes, we applied the additional filter criterion with a 2-fold difference in expression (Supplement 3, available at Carcinogenesis Online). The gene expression levels were visualized using a heatmap, with additional gene and sample clusters for the top 50 regulated genes, which showed all of the biological replicates clustered together (Figure 2A).

Seven candidate genes were chosen for further investigation based on their known prostate expression and their general connection to cancer. After VPA treatment of 2E PCa cells, the expression levels of the following four genes were decreased: chemokine (C-X-C motif) ligand 15 (Ccl15), which encodes for a small cytokine belonging to the CXC chemokine family; RCC1 and BTB domain-containing protein 2 (Rbth2), which encodes for a member of the RCC1-related GEF family; both transcript variants of ceruloplasmin (Cp), which is the major copper-carrying protein in the blood and leukemia inhibitory factor (Lif), which encodes for an interleukin 6 class cytokine. In contrast, the expression levels of the following three genes were strongly increased: ubiquitin C-terminal hydrolase L1 (Uch1), which encodes a de-ubiquitinating enzyme; receptor-type tyrosine-protein phosphatase-like N (Ptprn), which encodes a member of the protein tyrosine phosphatase family and cyclin D2 (Ccd2), which encodes a member of the highly conserved cyclin protein family.

![Fig. 1. VPA inhibits cell migration, invasion and proliferation of PCa cells. (A) Primary mouse 2E PCa cells were treated with VPA for 48h and transferred to a migration assay under constant VPA treatment for 24h. Cell migration was significantly decreased in a dose-dependent manner up to 51% for the highest concentration (10 mM) of VPA. (B) After pre-treatment with VPA for 24h, 2E cells were subjected to invasion assays in the presence of VPA for 48h. The inhibition of the invasiveness of 2E cells reached 52% for the lowest concentration of VPA (5 mM). However, treatment with a higher concentration of VPA (10 mM) did not show an additive effect. (C) The proliferation of 2E cells after increasing treatment concentrations of VPA for 144h was measured using an 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay. The proliferation decreased in a dose-dependent manner. (D) The primary 2E PCa cells were plated at a low density and treated with different concentrations of VPA. When clear colonies formed in the control-treated cells, the colonies were fixed and stained with hemalum/eosin and counted. There was a clear dose-dependent reduction of colony formation in the VPA-treated cells. The results are expressed as the mean ± SD of three (A and C), four (B) and six (D) independent experiments. ns, not significant; *P < 0.01, **P < 0.001, ***P < 0.0001.

The differential expression of all seven candidate genes was verified using qRT-PCR analyses, and VPA had a dose-dependent effect on regulating the expression of all seven candidate genes (Figure 2B and C).
The expression of the candidate gene cyclin D2 is specifically increased in primary PCa cells after treatment with VPA as a consequence of increased acetylation in the promoter region of the cyclin D2 gene.

The candidate gene cyclin D2 was chosen for further investigation due to its known deregulation in PCa patients. After treating murine 2E PCa cells with VPA, qRT–PCR studies verified a strong dose- and time-dependent increase of Ccnd2 expression (Figure 3A). A 5 mM VPA treatment for 24 and 72h increased the expression of Ccnd2 10-fold and 40-fold, respectively, in comparison with the control cells (Figure 3A). The levels of protein expression were confirmed using western blot analyses, which demonstrated the re-expression of cyclin D2 after VPA treatment, and we observed that untreated 2E cells showed only marginal cyclin D2 protein expression (Figure 3B).

Cyclin D2 belongs to the family of highly conserved D-type cyclins, including two other family members, namely cyclin D1 and D3; therefore, we investigated whether the changes in expression are specific for cyclin D2. Neither the expression of Ccnd1 (Figure 3C) nor the expression of cyclin D3 (Figure 3D) was significantly changed after VPA treatment of 2E PCa cells.

To analyze the mechanism behind re-expression of cyclin D2 after VPA treatment, the 2E cells were cultured in the presence of different HDACis. Treatment with TSA (250 nM and 1 µM), SAHA (1 and 5 µM), apicidin (1.6 and 5 µM) and NaB (1 and 5 mM) induced the expression of cyclin D2 in murine 2E PCa cells, which was determined using qRT–PCR (data not shown) and western blot analysis (Figure 4A). However, treatment with VPA, an in vitro inoperable form of VPA, did not show an effect on the cyclin D2 expression level (Figure 4B and C), and after 72 h, high concentrations of VPM (5 mM) had cytotoxic effects. However, changes in the cyclin D1 expression were not observed after treating 2E cells with all HDACis or VPM (Figure 4B and D).

The re-expression of cyclin D2 in cancer cells is presumably due to the HDACi activity of VPA; therefore, we analyzed the acetylation status of three regions in the promoter using a ChIP assay with an antibody against acetylated histone 3 at lysine 9. Three different primer pairs covering the promoter region of the cyclin D2 gene from −527 to −777 were used in subsequent qRT–PCR analyses. As presented in Figure 4E, there was a clear increase in the acetylation of the cyclin D2 promoter region after 2E PCa cells were treated with VPA.

Additionally, there was an increase in the acetylation of the promoter regions from the other two upregulated genes identified in the oligonucleotide microarray, namely Uchl1 and Ptprn, (Supplement 4, available at Carcinogenesis Online). In contrast, increased acetylation was not detected in the promoter regions of the two downregulated candidate genes, that is, Cp and Rbbt2 (data not shown).

The re-expression of cyclin D2 after VPA treatment results in cancer-cell-specific proliferation response

Untreated 2E PCa cells only showed marginal cyclin D2 expression; therefore, we investigated the cyclin D2 expression in several human cancers and in human and murine non-malignant cell lines. PCa cell lines and some colorectal cancer (CRC) cell lines have an overall decreased expression of cyclin D2. In contrast, the majority of non-malignant cell lines express cyclin D2 to a higher extent (Figure 5C). Therefore, we tested the response of 15 different cell lines to VPA treatment. After treatment with 5 mM of VPA for 72 h, the expression of cyclin D1 changed only slightly, and on average not more than 5-fold (Figure 5A). In contrast, the expression of cyclin D2 in the cell lines with low basal expression of cyclin D2 changed considerably, ranging from 30-fold for PC-3, up to 300-fold for SW620, 650-fold for DU145 and 2300-fold for LNCaP (Figure 5B). The expression of cyclin D2 in the cell lines with high basal expression changed only slightly, for example, in prostate stromal cells, NIH/3T3 or Caco-2. L cells demonstrated an exception: even with considerable cyclin D2 basal expression, they showed a strong upregulation of cyclin D2 expression after VPA treatment (Figure 5B and C). In most cases, the effects detected in all cell lines using qRT–PCR were confirmed using western blot analysis (Figure 5C).
VPA and cyclin D2 re-expression

To clarify the connection between cyclin D2 re-expression in cancer cells and the proliferation response, we performed proliferation assays on all of the cell lines. As depicted in Figure 5C and D, there is a clear link between cyclin D2 basal expression and proliferation after VPA treatment. Almost all of the fibroblast cell lines showed a high basal expression of cyclin D2, and after VPA treatment they did not show an increase in expression. Consequently, they responded to VPA treatment with only a mild inhibition of proliferation (Figure 5D).

In CRC, the connection is different; SW620 cells responded to the VPA treatment with strong re-expression of cyclin D2, but only mild inhibition of proliferation (Figure 5D).

Importantly, the PCa cell lines all re-expressed cyclin D2 after VPA treatment, and they all showed strong inhibition of proliferation (Figure 5D).

To further strengthen the observed correlation between cyclin D2 expression and proliferation, we inhibited cyclin D2 expression via siRNA in NIH/3T3 fibroblasts. An efficient downregulation was achieved using siRNA B (Figure 6A). This inhibition of cyclin D2 expression led to a significant 20% increase in cell proliferation (Figure 6B).

**Cyclin D2 expression is lost in human PCa**

Recent publications have demonstrated that cyclin D2 RNA levels are eliminated during PCa development, most likely due to promoter methylation (22,23). These studies used RT–PCR on microdissected material; therefore, we investigated the expression of cyclin D2 using immunohistochemistry to monitor cyclin D2 on the cellular basis. We included differentiated tumors (n = 15) and one lymph node metastasis, as well as normal prostate tissue (n = 6), in our patient cohort. We determined that cyclin D2 is expressed in the proliferating epithelium of the normal prostate (Figure 6C), which shows only a few Ki67-positive cells (own observations and refs (33,34)). Moreover, tumor tissues showed a complete loss of cyclin D2 expression, although they were positive for Ki67 expression; whereas interstitial cells showed high levels of cyclin D2 expression and therefore served as an internal positive control (Figure 6E and F). No cyclin D2 expression was detected in the stained lymph node metastases either, indicating a role for cyclin D2 as a tumor suppressor in PCa (Figure 6D). Notably, staining the same tissue specimens for cyclin D1 verified its overexpression and supported its opposing role as an oncogene (Figure 6G and H).

**Discussion**

In this study, we demonstrated that the HDACi VPA is a useful drug for the treatment of PCa and other cancer entities. In a murine in vitro model system for PCa, we demonstrated that cell migration, invasion and proliferation are diminished after VPA treatment. Global gene expression profiling revealed that several interesting genes were deregulated, one of which, cyclin D2, was subjected to further investigation. The cyclin D2 expression was the only member of the D-type cyclins to be upregulated after VPA treatment. The specific increase of cyclin D2 expression was also achieved using several other HDACis; the re-expression of Ccnd2 and two other upregulated genes, Uchl1 and Ptprn, was proven to be the result of increased acetylation in the promoter regions of these genes. This increased cyclin D2 expression after VPA treatment was dependent on the basal cyclin D2 expression levels. Therefore, several other cell lines (non-malignant fibroblasts and PCa as well as CRC cells) were tested for their response to
VPA. The cell lines, with either low or no basal cyclin D2 expression, reacted to VPA treatment with a strong re-expression of cyclin D2, which resulted in a strong inhibition of proliferation.

Migration and invasion are characteristics of highly malignant cancer cells and indicate the metastatic potential of these cancer cells. Our murine 2E PCa cells represent an aggressive cell line and, more importantly, treatment of the 2E cells with VPA sufficiently suppressed these attributes. In these cells, VPA treatment caused a reduction in migration and invasion, which contrasts the results reported by Chen et al. (35) showing that there was no influence of VPA on PCa cell lines, LNCaP, PC-3 and DU145. Our results were confirmed for the PC-3 cells by different groups previously and during these studies (11,15). Additionally, these studies show that VPA treatment increases the expression of E-cadherin and changes the expression level of several integrins and cell adhesion molecules.

The impact of VPA on the proliferation of cancer cells from myeloid and lymphoid leukemia as well as from numerous solid tumors is well established (reviewed in (17,36)). After VPA treatment, decreased cell proliferation in LNCaP and PC-3 has been previously reported (12–14,16). In this study, we verified these findings in PC-3 and also showed the inhibition of proliferation in our murine in vitro model of PCa.

To determine which molecular targets are the promoter for the cellular effects observed in VPA-treated 2E cells, gene expression profiling experiments were performed. To identify the primary transcriptional effects of the increased promoter acetylation, these analyses were conducted using a relatively low concentration of VPA (1 mM) for 24 h, which adequately increased histone 3 acetylation in 2E PCa cells. Several candidate genes were found to be differentially expressed (Supplement 3, available at Carcinogenesis Online). Of the downregulated genes, Ccnd1, is frequently upregulated in patients with PCa and is a potential new marker for the detection of PCa (37).

Another candidate gene identified in this study was cyclin D2, which was strongly upregulated after treating 2E PCa cells with VPA. As a family member of the D-type cyclins, cyclin D2 is a key player in the cell cycle, which promotes the progression from the G1 phase to the S phase (18). Cyclin D2 is overexpressed in several cancers, including colon cancer and gastric cancer, which led to the hypothesis that cyclin D2 acts as a proto-oncogene (20,38,39). In contrast, almost all breast cancer cell lines analyzed were deficient in cyclin D2 expression, whereas normal epithelial breast cells express cyclin D2 (40,41). Similar results were obtained in lung cancer cell lines and the loss of cyclin D2 expression in breast, pancreatic and gastric...
VPA and cyclin D2 re-expression

cancers (41–44). Consistent with these findings, the \textit{CCND2} gene is hypermethylated and thereby inactivated in PCa patient specimens (23, 24).

Moreover, Kobayashi et al. (24) demonstrated that overexpression of cyclin D2 in LNCaP PCa cells leads to the inhibition of proliferation in a cyclin-dependent kinase–independent manner, which suggests that cyclin D2 functions as a tumor suppressor and has additional roles in the cell apart from being a cyclin-dependent kinase binding partner. In this study, we demonstrate that cyclin D2 is the only D-type cyclin family member to be re-expressed in PCa cells after VPA treatment.

We chose VPA as the HDACi for our studies because this drug has a long history in medicine and has been used for many years in the clinic. Recently, another study also highlighted VPA’s ability to reduce the risk of PCa in epilepsy patients treated with VPA (45). Nevertheless, we also tested other HDACis to ascertain whether re-expression of cyclin D2 was specific to VPA or to HDACis in general.

Here, we found that cyclin D2 was also re-expressed after treating
D.Witt et al.

2E PCa cells with other HDACis. In contrast, the in vitro inoperative form of VPA, namely VPM, had no effect on cyclin D2 expression. These results suggest that re-expression of cyclin D2 is dependent on the histone acetylation of the cyclin D2 gene promoter region. Subsequent ChIP experiments also ascertained the increased acetylation of the cyclin D2 promoter after VPA treatment, which was also found for the other two genes that were upregulated after VPA treatment in 2E cells.

The re-expression of cyclin D2 after VPA treatment was not only observed in 2E PCa cells but also observed in three other established PCa cell lines and one CRC cell line. Non-malignant cell lines with a high basal expression of cyclin D2 did not show a strong increase in cyclin D2 expression after VPA treatment. Because fibroblasts have a high basal expression level of cyclin D2, we concluded that VPA restores this expression from a previously inactivated cyclin D2 promoter, as opposed to generally inducing the overexpression of cyclin D2. When comparing the expression data and the proliferation data, we observed a clear dependence between cyclin D2 re-expression after VPA treatment in the various cell lines tested and an inhibition of proliferation. After VPA treatment, increased cyclin D2 expression correlated with an increase in the inhibition of proliferation. This result suggests a new role for cyclin D2 as a tumor suppressor gene, which is controversial in the literature; however, several other reports reinforce this hypothesis (22, 24, 46). We, therefore, performed immunohistochemical analyses on tissue sections of 21 patients containing normal prostate tissue and differentiated PCa. Although all normal prostate displayed expression of cyclin D2 in proliferating cells of prostate epithelium, no cyclin D2 expression was observed in 12 out of 15 PCa specimens; however, high Ki67 positivity was detected in these specimens. These results further strengthen our hypothesis of cyclin D2 acting as a tumor suppressor in PCa. The clear correlation between the re-expression of cyclin D2 and the inhibition of proliferation after VPA treatment in PCa cell lines was not reproducible in the colorectal cancer cell lines. SW620 responded with a high re-expression of cyclin D2 after VPA treatment, but the proliferation was only marginally affected. We attribute this behavior to the fact that in CRC, cyclin D2 represents an oncoprotein because human CRC specimens display an overexpression of cyclin D2 (38).

To further investigate whether cyclin D2 re-expression is responsible for the observed inhibition of proliferation, we performed immunohistochemical analyses on tissue sections of 21 patients containing normal prostate tissue and differentiated PCa. Although all normal prostate displayed expression of cyclin D2 in proliferating cells of prostate epithelium, no cyclin D2 expression was observed in 12 out of 15 PCa specimens; however, high Ki67 positivity was detected in these specimens. These results further strengthen our hypothesis of cyclin D2 acting as a tumor suppressor in PCa. The clear correlation between the re-expression of cyclin D2 and the inhibition of proliferation after VPA treatment in PCa cell lines was not reproducible in the colorectal cancer cell lines. SW620 responded with a high re-expression of cyclin D2 after VPA treatment, but the proliferation was only marginally affected. We attribute this behavior to the fact that in CRC, cyclin D2 represents an oncoprotein because human CRC specimens display an overexpression of cyclin D2 (38).

To further investigate whether cyclin D2 re-expression is responsible for the observed inhibition of proliferation, siRNA-mediated downregulation experiments were performed in 2E cells (data not shown). Upon treatment with VPA, transfection with cyclin D2-specific siRNAs was not sufficient to inhibit the strong upregulation of cyclin D2 expression. To date, there is no specific inhibitor for cyclin D2 available, which is not inhibiting cyclin D1 and D3. Thus, we did not directly correlate cyclin D2 re-expression and the inhibition of 2E cell proliferation. Instead, we downregulated cyclin D2 expression using siRNAs in NIH/3T3 fibroblasts, which increased cell proliferation after efficient cyclin D2 knockdown.
These findings support the hypothesis that cyclin D2 acts as a tumor suppressor in PCa. Therefore, VPA is a potential anticancer therapeutic option for cancers with epigenetically repressed cyclin D2 expression.

To date, there is no evidence of a direct correlation of cyclin D2 deficiency and higher invasiveness in the literature. Earlier studies showed that cyclin D2 deficiency caused by promoter hypermethylation is a general effect in the development of breast cancer of different subtypes (41). Recently, Swift-Scanlan et al. (47) observed a direct correlation between cyclin D2 promoter methylation and non-metastatic breast cancer.

In summary, our work highlights the new function for the formally known antiepileptic drug VPA as a potent therapeutic strategy against PCa, even for cancers in highly aggressive states. We also determined that the molecular mechanism by which VPA inhibits the proliferation of cancer cells is through the re-expression of cyclin D2.

Supplementary material
Supplement 1–4 can be found at http://carcin.oxfordjournals.org/

References

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