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Intra-Tumoral Delivery of shRNA Targeting Cyclin D1 Attenuates Pancreatic Cancer Growth

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Abstract

The aim of this study was to assess the biological consequences of cyclin D1 silencing in pancreatic cancer cells. A replication-defective lentivirus based small hairpin RNA (shRNA) system targeting cyclin D1 caused a marked reduction in cyclin D1 protein levels in ASPC-1 and BxPC3 pancreatic cancer cell lines in conjunction with decreased cell growth and invasiveness *in vitro*. Moreover, a single intratumoral injection of the recombinant lentivirus targeting cyclin D1 attenuated the growth of pre-existing tumors arising from two distinct cell lines. This attenuated growth correlated with decreased proliferation and angiogenesis, and attenuated VEGF expression. It is concluded that lentivirus-delivered shRNA targeting cyclin D1 suppresses the growth, invasiveness, tumorigenicity and pro-angiogenic potential of human pancreatic cancer cells, raising the possibility that intra-tumoral injections of viruses targeting cyclin D1 could provide a novel therapeutic approach in pancreatic ductal adenocarcinoma.

Keywords

cyclin D1; pancreatic cancer; shRNA; lentivirus

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States 1. In spite of recent therapeutic advances, long term survival in PDAC is often limited to patients who have had surgery in early stage of the disease 2. The biological aggressiveness of PDAC is due, in part, to the tumor's resistance to chemotherapy and to its propensity to metastasize even when the primary tumor is small 3. PDAC is also characterized by a high frequency of mutations in the Kras gene, as well as by the inactivation of several tumor suppressor genes such as p16^{Ink4a}, p53 and Smad4 4. PDAC also displays abnormal upregulation of multiple mitogenic and angiogenic growth factors and their cognate high affinity receptors 5. Together, these alterations serve to enhance the biological aggressiveness of PDAC.

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Cyclin D1, a cell cycle regulator, is known to be overexpressed in several human cancers including PDAC 6. In response to mitogenic growth factors, normal cells exhibit cyclin D1 accumulation by early to mid phase of G1. Cyclin D1 then activates its cognate cyclin dependent kinases, CDK4 and 6. Activated CDK4/6 initiates the phosphorylation-dependent inactivation of the retinoblastoma protein (RB) which then promotes progression through the G1-S phase of the cell cycle 7. Cyclin D1 also has CDK-independent functions 8. Thus, it can associate with and enhance the activity of transcription factors by recruiting cofactors that govern chromatin remodeling 9, and contribute to the regulation of cell metabolism, differentiation and migration 7.

PDACs express high levels of cyclin D1, and this overexpression has been correlated with decreased patient survival 10. Moreover, suppression of cyclin D1 expression by an antisense-based strategy resulted in marked inhibition of pancreatic cancer cell growth *in vitro* and *in vivo* 11, 12. However, such an antisense strategy is not readily amenable for use in therapeutic regimens. RNA interference (RNAi) has rapidly become a powerful tool for gene silencing, drug discovery and target validation 13, and vectors that synthesize small hairpin (sh) RNA permit sustained gene silencing 14. These vectors allow for the synthesis of 50 base pair (bp)-long single stranded RNAs that fold in 21–23 bp dsRNA with a small hairpin in the middle, and that are subsequently processed to siRNAs by the cellular machinery. These shRNA expression vectors can be engineered to contain selectable markers to generate stable transfectants, to co-express reporter genes, and to be incorporated into viral vectors such as a lentiviral vector 15. This virus is modified to be non-replicative, and is efficiently transduced into human cells, including non-dividing cells 15, 16. It then integrates into the host genome, resulting in long-term and stable expression of the shRNA 17.

In the present study we used third generation lentiviral vectors to integrate the sequence of a cyclin D1 shRNA in two human pancreatic carcinoma cell lines, thereby allowing for stable expression of the shRNA and a sustained suppression of cyclin D1 expression. This down-regulation resulted in attenuated growth of the cells in anchorage dependent and independent conditions, as well as decreased invasiveness *in vitro*. Moreover, a single intra-tumoral injection of these recombinant lentiviruses targeting cyclin D1 attenuated the growth of pre-existing tumors by decreasing cancer cell proliferation and angiogenesis.

Materials and Methods

Cell growth and invasion assays

ASPC-1 and BxPC3 human pancreatic cancer cell lines from ATCC (Manassas, VA) were grown in RPMI 1640 (Mediatech Inc., Herndon, VA). Medium was supplemented with 10% fetal bovine serum (FBS) from Omega Scientific Inc. (Tarzana, CA), and with 100 U/ml penicillin and 100µg/ml streptomycin (complete medium).

Doubling time

To assess doubling time, cells were seeded in 6 well plates at a density of 1.0×10^4 cells/plate and doubling time was calculated over 5 days. All doubling time experiments were repeated three separate times, each time on separate days.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) growth assay

Cells (3,000 per well) were plated in 96-well microtiter plates (Corning, Acton, MA). MTT was added to each well (final concentration 0.5 mg/ml) 72 hours later. The plates were incubated at 37°C for 3 hours, and the medium replaced with 100 μ l of acidified isopropanol followed by gentle shaking for 15 min to solubilize the formazan blue crystals. Absorbance at 570 nm was measured using a microtiter plate reader. Cell viability was expressed as the percentage of growth of parental cells. Each experimental condition was assayed in six wells, and each experiment was repeated at least three times.

Soft Agar Assay

Soft agar assays were performed as reported previously 18. Cells (8×10^3) were suspended in complete medium containing 0.3% agar and seeded in triplicate in 6-well plates onto a base layer of complete medium containing 1% agar. Complete medium containing 0.3% agar was added every 5 days for 15 days, and colony counting was then performed.

Invasion assays

Invasion assays were performed as reported previously 19. Briefly, cells were suspended in 500 μ L RPMI with 0.1% bovine serum albumin and placed onto the upper compartment of Matrigel-coated Transwell chambers (8 μ m pore size, BioCoat Matrigel Invasion Chambers; BD Biosciences). The lower compartment was filled with 750 μ L RPMI with 5% serum. After 18 to 20 h, cells on the upper surface of the filter were carefully removed with a cotton swab and the membranes were fixed in methanol. The cells that had migrated through the membrane to the lower surface of the filter were stained with toluidine blue (Fisher Scientific) and counted using a light microscope.

Immunoblotting

Immunoblotting was done as described previously 20. Briefly, PVDF membranes were incubated overnight with a 1:1000 dilution of anti-cyclin D1 human anti-mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA), washed, and incubated for 30 minutes with a secondary horseradish peroxidase-conjugated antibody. Bound antibodies were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). To confirm equal loading of lanes, membranes were stripped for 30 minutes at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol and blotted with a 1:10,000 dilution of a rat anti-tubulin antibody (Abcam, Cambridge, MA).

siRNA transient transfection and lentivirus shRNA gene transduction

siRNA sequences directed against human cyclin D1 (Dharmacon, Lafayette, CO) were initially transfected into ASPC-1 cells using Jet PEI (Qbiogene, Solon, OH) according to the manufacturer's protocol. Oligonucleotides corresponding to the shRNA sequence of interest

were annealed and cloned into the lentivirus vector, pLentiLox 3.7 (pL3.7) (Addgene, Cambridge, MA). Virus stocks were prepared by co-transfecting pL3.7 with three packaging plasmids (pMDLg/pRRE, CMV-VSVG and RSV-Rev) into 293T cells 21. The viral supernatants were harvested 36–48 hours later, filtered and centrifuged (90 min at 25,000 × g). Viral titers were determined by fluorescence-activated cell sorting (FACS) analysis.

In vivo tumorigenicity assay

To assess the effects of cyclin D1 suppression on established tumors, 24 mice were injected subcutaneously into the dorsal flank area with 200 µl containing 1×10^6 ASPC-1 cells. Separately, 24 mice were similarly injected with 200 µl containing 1×10^6 BxPC3 cells. The mice were divided randomly into three groups of 8 mice per cell line. Once tumors reached a volume of 30–40 mm³ (usually 8–10 days after injection of the cells), the tumors were injected with 50 µL (4.10^7 viral particles) of Optimem (Invitrogen, Carlsbad, CA), with virus containing the shRNA against luciferase, or with virus containing the shRNA against cyclin D1. The tumors were measured every three days. Tumor volumes were calculated as $\pi/4 \times \text{width} \times \text{height} \times \text{length}$ of the tumor 22. When the tumor diameter reached 15 mm, the mice were sacrificed.

Immunohistochemistry

Following rapid tumor removal, tissues were cryo-embedded in cryo-OCT compound (Fisher Scientific, Pittsburgh, PA). All immunohistochemistry experiments were done as previously described 23. The following antibodies were used: a rabbit polyclonal anti-Ki-67 (Abcam, Cambridge, MA; 1:50 dilution) antibody to assess proliferation, a rat anti-mouse monoclonal antibody targeting CD31 as an endothelial cell marker (PharMingen, San Jose, CA; 1:100 dilution), and a polyclonal anti-VEGF (Santa Cruz, Santa Cruz CA; 1:50 dilution) antibody. Quantitative morphometric analysis of Ki-67, CD31 and VEGF positive cells in tumor samples was carried out as previously reported 24. Briefly, 10 areas/slide were captured using Olympus DP70 camera (100X magnification) and quantified with the Image-Pro plus program (Version 4.51, Media cybernetics, L.P., Silver Spring, MD). Three tumors per group were analyzed. Blood vessel densities were calculated as the ratio of CD31 positive areas to the total area of the image, whereas proliferation was calculated as the ratio of Ki-67 area to total cell area.

Statistical analysis

Data were analysed using the Kruskal and Wallis tests for mean comparisons using the Dunn-Benferroni test for multiple comparisons, with $p < 0.05$ taken as significant.

Results

shRNA selection and knock down of cyclin D1

To select the shRNA sequence to be introduced into the lentivirus, a pool of different siRNA sequences directed against human cyclin D1 were tested in ASPC-1 cells. After siRNA transfection, cell lysates were subjected to immunoblotting using anti-cyclin D1 antibodies. The siRNA pool efficiently silenced cyclin D1 expression at the protein level (data not shown). Each sequence of this pool was then tested in order to select the two most efficient

siRNA sequences, which were then used to design the oligonucleotides for the shRNA targeting cyclin D1. These oligonucleotides were then annealed and cloned into the pL3.7 vector.

Lentivirus infectivity, determined following infection with the GFP-expressing lentivirus at a multiplicity of infection (m.o.i.) of 10, revealed that 90% of ASPC-1 and BxPC3 human pancreatic cancer cells exhibited strong fluorescence, and these results were confirmed by FACS (data not shown). Next, ASPC-1 and BxPC3 were infected at different m.o.i. with shRNA-lentiviruses targeting cyclin D1, or with shRNA-lentiviruses targeting luciferase (shLuc-LV) that was used as a control. We tested the processing and subsequent functionality of the control shRNA by infecting 293 cells that constitutively express luciferase with shLuc-LV. A decrease in luciferase activity in these cells demonstrated that the shRNA targeting luciferase is processed by the silencing pathway (data not shown).

As determined by immunoblotting, shRNA sequences 8 and 10 were the most effective at suppressing cyclin D1 levels in both cell lines, as was the combination of both shRNAs (Figure 1). These viruses were therefore used in all subsequent experiments.

Cyclin D1 down regulation results in reduced proliferation and invasion

Initial experiments were performed to determine the effects of cyclin D1 down regulation on cell doubling times. Parental and mock-infected cells had similar doubling times of 31 ± 0.5 h, whereas ASPC-1 cells infected with lentivirus expressing shRNA 8 (sh8-LV) and shRNA 10 (sh10-LV) displayed doubling times of 39 hours ($p < 0.05$) and 40 ± 1.34 h ($p < 0.01$), respectively. Similarly, parental and mock infected BxPC3 cells exhibited doubling times of 25 ± 0.7 h, whereas infection of these cells with sh8-LV and sh10-LV prolonged the doubling times to 34 ± 1.4 h ($p < 0.05$) and 30 ± 1.2 h ($p < 0.05$), respectively.

Cyclin D1 down regulation was also associated with a significant inhibitory effect on cell proliferation, as determined by the MTT assay (Figure 2a), and by a significant decrease in colony formation in soft agar (Figure 2b). Thus, as shown in parenthesis by the means \pm standard errors and respective p values, by comparison with control, the number of colonies formed was decreased by 54% (197 ± 5 ; $p < 0.01$) and 51% (203 ± 2 ; $p < 0.01$) in ASPC-1 cells infected with sh8-LV and sh10-LV, respectively, and by 54% (190 ± 4 ; $p < 0.01$) following infection with both viruses. Moreover, in BxPC3 cells, colony number was decreased by 33% (297 ± 4 ; $p < 0.05$) and 36% (287 ± 9 ; $p < 0.05$) following infection with sh8-LV and sh10-LV, respectively, and by 43% (252 ± 9 ; $p < 0.01$) following infection with both viruses.

The effects of cyclin D1 knock-down on cell invasion were examined next, using a Matrigel-based invasion assay. In the presence of serum (5%), cell invasion was enhanced in ASPC-1 and BxPC3 cells. In both cell lines, infection with sh8-LV, sh10-LV or the combination significantly attenuated the stimulatory effects of serum by comparison with values observed in mock-infected cells (Figure 2c). Thus, invasion was attenuated by 39% (41.5 ± 1.3 ; $p < 0.01$) and 50% (34.2 ± 1.5 ; $p < 0.01$) in ASPC-1 cells infected with sh8-LV and sh10-LV, respectively, and by 57% (29.3 ± 1.3 ; $p < 0.01$) following infection with both viruses. Similarly, invasion was attenuated by 56% (59.2 ± 4.9 ; $p < 0.01$) and 47% ($70.7 \pm$

–4.7; $p<0.01$) in BxPC3 cells infected with sh8-LV and sh10-LV, respectively, and by 54% (61.2 ± 4.2 ; $p<0.01$) following infection with both viruses. These observations indicate that in both cell lines, the combination of sh8-LV and sh10-LV was at least as effective as either lentivirus alone.

Cyclin D1 knock down attenuates the growth of established tumors

To determine whether the lentivirus could efficiently infect tumors and attenuate their growth, ASPC-1 and BxPC3 cells, which express high cyclin D1 levels (data not shown), were used next. Tumors were generated by subcutaneous injections of ASPC-1 or BxPC3 cells (1×10^6 cells/injection). Upon reaching a volume of 30–40 mm³, intra-tumoral injections were initially performed with a GFP-expressing p13.7 virus. After one week, strong GFP expression was observed in the dissected tumors, which was similar to that observed following injection of cancer cells that were infected *ex-vivo* with the GFP-expressing p13.7 virus (supplemental data, figure S1). These observations indicate that following injection into an established tumor, the virus was able to diffuse and infect cells throughout the tumor mass. The same procedure was used next for the shRNA experiments.

Each tumor was injected once with 50 μ l of a combination of sh8-LV and sh10-LV (shD1-LV), or with 50 μ l of a control lentivirus expressing shRNA directed against luciferase (shLuc-LV). When experiments were terminated for tumor harvesting, the ASPC-1- and BxPC3-derived tumors that were infected with shD1-LV exhibited a 44% (115 ± 18 ; $p<0.05$) and a 65% (230 ± 18 ; $p<0.05$) decrease in volume, respectively, by comparison with corresponding controls (Figure 3, and supplemental data, Figure S2). A statistically significant difference in tumor volumes between shLuc-LV-injected tumors and shD1-LV-injected tumors was seen as early as 9 and 18 days following viral injection of ASPC-1- and BxPC3-derived tumors, respectively. Moreover, the subsequent growth of all shD1-LV-injected tumors remained significantly attenuated.

ASPC-1-derived tumors injected with shD1-LV exhibited a 47% decrease ($p<0.01$) in Ki-67 immunoreactivity (a marker of cell proliferation), and a 56% decrease ($p<0.05$) in CD31 immunoreactivity (an endothelial cell marker), by comparison to respective controls (Figure 4). Similarly, BxPC3-derived tumors exhibited a 42% decrease ($p<0.05$) in Ki-67 immunoreactivity and a 45% decrease in CD31 immunoreactivity ($p<0.05$), by comparison with respective controls (Figure 5). In addition, VEGF immunoreactivity in ASPC-1- and BxPC3-derived tumors injected with shD1-LV was decreased by 52% ($p<0.01$) and 58% ($p<0.01$), respectively, by comparison with corresponding controls (Figures 4–5).

Discussion

The three D-type cyclins are encoded by distinct genes, exhibit considerable amino acid sequence homology, and have both overlapping and separate functions. The cyclin D1 gene, in particular, has been associated with neoplastic transformation. This gene is located on chromosome 11q13, a region that is amplified in breast, bladder, head and neck, lung and esophageal cancers 25, 26, and that exhibits chromosomal translocations and rearrangements in parathyroid adenomas and B cell lymphomas 25, 26. The associated increase in cyclin D1 levels has been correlated with a shortened G1 phase of the cell cycle and reduced mitogen

dependency 27–29. Cyclin D1 inhibition by antibody microinjection or by transfection with a cyclin D1 antisense expression construct prevents normal fibroblasts from entering the S phase of the cell cycle and markedly inhibits the proliferation of human esophageal and colon cancer cells 30–32, and murine lung cancer cells 33. Moreover, increased cyclin D1 expression correlates with decreased survival in PDAC 6, 10, as well as in esophageal 34 and breast 35 cancers. Although there are fewer functional data for the two other members of the cyclin D family, cyclin D3 expression is also upregulated in PDAC 36 while cyclin D2 expression was reported to be reduced as a result of promoter methylation 37.

Lentiviruses are a subclass of retroviruses that allow for efficient gene transduction into cells and tissues, infecting both proliferating and non-proliferating cells, and resulting in stable integration of specific genes into the cellular genome 38. In contrast to adenoviruses, lentiviruses do not trigger a potentially dangerous immune response. They are ideally suited, therefore, for use in silencing strategies in cancer cells 14. In the present study we determined that a single exposure of ASPC-1 and BxPC3 human pancreatic cancer cell lines to lentiviruses encoding two different specific shRNA targeting cyclin D1 resulted in stable integration of the virus and high level of expression of the shRNAs, along with subsequent marked reduction in cyclin D1 protein levels. Thus, lentiviral delivery of shRNA is an efficient way to markedly down-regulate cyclin D1 in pancreatic cancer cells.

Cyclin D1 down-regulation was associated with decreased cell proliferation, attenuated anchorage-independent growth, and blunted invasiveness *in vitro*. This is the first direct demonstration that cyclin D1 promotes invasion in pancreatic cancer cells. This finding is in agreement with the observation that cyclin D1 overexpression in glioma cells is associated with increased matrix metalloproteinase-2 (MMP-2) and MMP-9 activity and up-regulation of Rac1, which modulates the formation of ruffling membranes and cell motility 39. Moreover, in mouse embryo fibroblasts, mammary epithelial cells and macrophages derived from cyclin D1^{-/-} mice, migration is greatly attenuated 40, 41. These observations may explain why patients with cyclin D1-positive pancreatic tumors tend to experience perineural and lymphatic invasion more often than patients with cyclin D1-negative tumors 42, 43, and suggest that cyclin D1 may contribute to the invasiveness and metastatic potential of pancreatic cancer cells.

To test the therapeutic potential of the cyclin D1 shRNA expressing lentivirus approach, direct injection of the recombinant lentivirus was performed in established tumors. A single injection of the recombinant lentivirus was sufficient to inhibit the growth of established xenografts arising from either ASPC-1 or BxPC3 cells, indicating that down-regulation of cyclin D1 by the shRNA lentiviral strategy resulted in a sustained biological effect *in vivo*. This conclusion is also supported by the observation that Ki-67 staining was significantly decreased in these tumors. Inasmuch as Ki-67 is a marker of actively proliferating cells, this observation indicates that cyclin D1 levels directly affect proliferation status *in vivo*. This decrease in proliferation is consistent with the known effects of cyclin D1 on the cell cycle, with our *in vitro* observations with respect to cell growth, as well as with our observation that cyclin D1 antisense expression can block the mitogenic action of EGF, FGF-2 and IGF-I in pancreatic cancer cells 11.

Down-regulation of cyclin D1 was also associated with a significant attenuation in tumor angiogenesis, as evidenced by decreased CD31 immunoreactivity and measured by quantitative morphometry. Given our finding that VEGF levels were decreased in these tumors, it is possible that silencing cyclin D1 led to decreased VEGF expression, which may have contributed to decreased tumor angiogenesis. In support of this conclusion, cyclin D1 down-regulation in a colorectal cancer cell line resulted in lower levels of VEGF expression *in vitro*, which was associated with decreased tumor angiogenesis *in vivo* 12. It is also possible, however, that the intra-tumoral injection of our lentiviruses led to direct down-regulation of cyclin D1 in tumor endothelial cells and a direct suppressive effect on angiogenesis in addition to the lowered expression of VEGF. Irrespective of the mechanisms, given the importance of tumor angiogenesis in PDAC 23, our results raise the possibility that targeting cyclin D1 in PDAC may have the additional benefit of suppressing angiogenesis in this malignancy.

Our observations also point to the exciting possibility that this recombinant lentivirus could eventually be delivered by endoscopic ultrasonography into established tumors in patients with PDAC, thereby yielding a novel therapeutic approach for this deadly malignancy. Several lines of evidence suggest that such an approach may have multiple beneficial effects. First, increased cyclin D1 expression is observed relatively early during tumorigenesis, in pre-malignant lesions termed Pancreatic Intraepithelial Neoplasia (PanIN), raising the possibility that targeting cyclin D1 could prevent the progression toward higher grade lesions and cancer. This is a potentially important aspect of such therapy since the presence of PanIN lesions in the vicinity of the tumor resection margins may serve as a source for cancer recurrence in PDAC. Second, cyclin D1 is required for carcinogenesis in some cancer models, as cyclin D1-deficient mice do not form mammary carcinomas despite the induction by *c-neu* and *v-Ha-ras* 43. Therefore, targeting cyclin D1 may block the actions of oncogenic Kras, which is commonly mutated in PDAC, and the actions of multiple tyrosine kinase receptors which are often overexpressed in PDAC 5. Third, PDAC is characterized by the presence of an abundant stroma that contains proliferating cancer associated fibroblasts and pancreatic stellate cells, and this stroma has been proposed to confer a growth advantage to pancreatic cancer cells within the tumor mass 44. Direct injections of the virus into the pancreatic tumor mass would result in a down regulation of cyclin D1 in non cancer cells as well, which would suppress the proliferation of stromal elements. Fourth, suppression of cyclin D1 levels could result in decreased angiogenesis through direct actions on the endothelial cells, and, indirectly, by decreasing VEGF expression.

Elevated cyclin D1 levels contribute to the chemoresistance of pancreatic cancer cells, as shown by the increased chemosensitivity to cis-platinum of PDAC cells when cyclin D1 expression is inhibited 11, 45. Therefore, targeting cyclin D1 may allow for suppression of tumor growth and angiogenesis, while allowing for more effective responses to chemotherapeutic agents. Taken together, these observations raise the possibility that intra-tumoral injections of viruses targeting cyclin D1, in conjunction with standard chemotherapy, could provide a novel therapeutic approach in PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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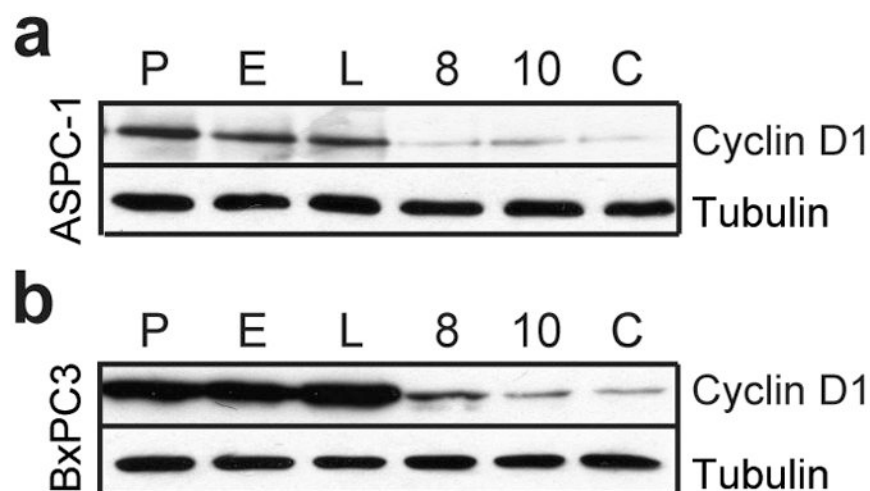


Figure 1. Cyclin D1 silencing

ASPC-1 (a) and BxPC3 (b) cells that were either not infected (parental cells: P) or infected with the mock-infected lentivirus pll37 (M), or with pll37 containing sequences encoding luciferase (L), cyclin D1 specific shRNA 8 (8) or 10 (10), or their combination (K). Cell lysates, prepared 72 h after infection, were subjected to immunoblotting using anti-cyclin D1 antibodies. Membranes were re-probed with anti-tubulin antibody to assess equivalent lane loading.

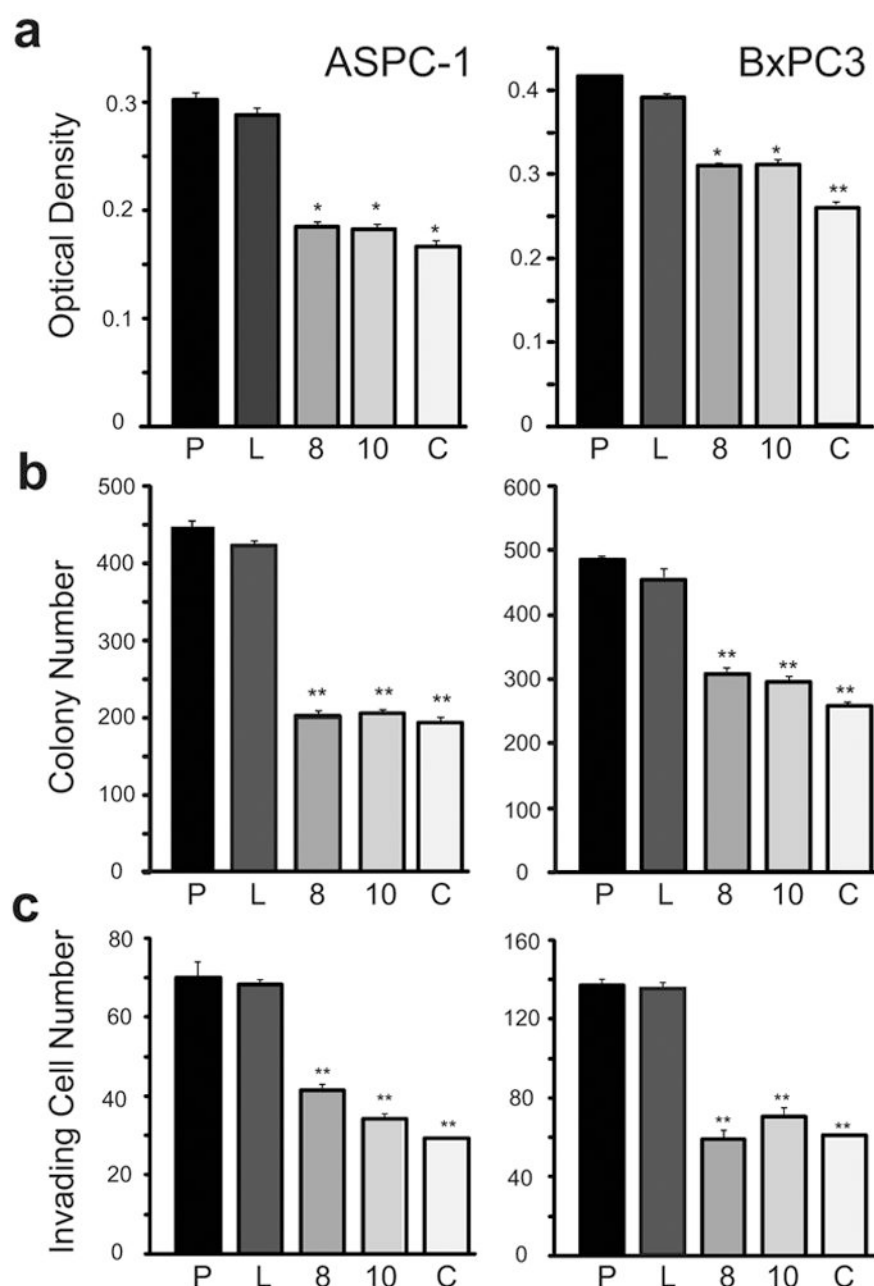


Figure 2. In vitro consequences of cyclin D1 silencing

ASPC-1 and BxPC3 cells that were either not infected (parental cells: P) or infected with the mock-infected lentivirus pL37 (E), or pL37 containing sequences encoding luciferase (L), or cyclin D1 specific shRNA 8 (8) or 10 (10), or their combination (K). **(a)** Anchorage-dependent growth was determined by the MTT assay. Data are the means \pm SE of triplicate determinations from three independent experiments. * $p < 0.05$, ** $p < 0.01$ when compared with Luc. **(b)** Anchorage-independent growth was determined in soft agar. Data are the means \pm SE of triplicate determinations from three independent experiments. ** $p < 0.01$ when compared with Luc. **(c)** Cell invasion was determined in Transwell chambers. Data are

the means \pm SE of triplicate determinations from three independent experiments. **p<0.01 when compared with Luc.

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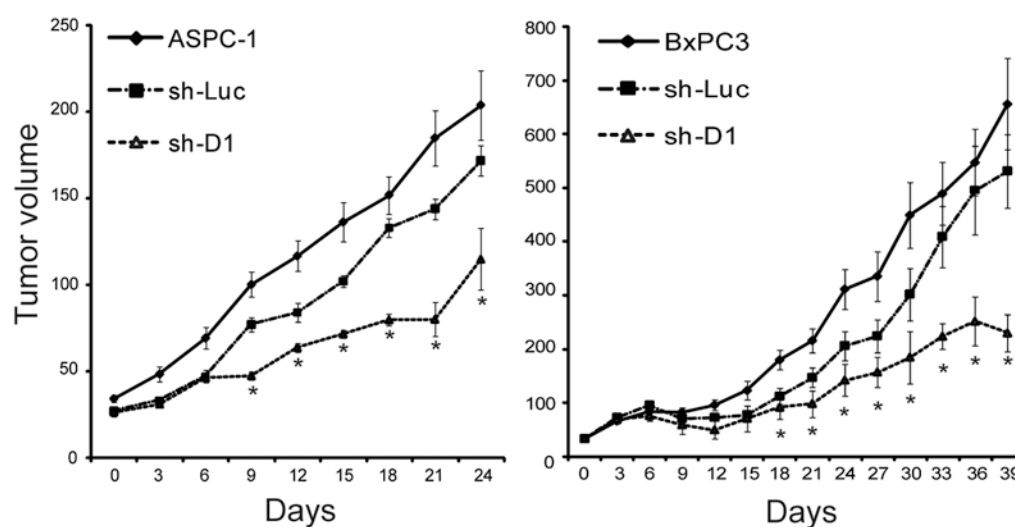


Figure 3. Effects of cyclin D1-directed shRNA on tumor growth

ASPC-1 or BxPC3 cells were injected subcutaneously in athymic nude mice. After tumors reached a volume of 30–40 mm³, they were either injected with buffer (ASPC-1) or with luciferase-directed (sh-Luc) or cyclin D1-directed (sh-D1) shRNA-lentiviruses. Tumor volumes were calculated in mm³ as described in the Methods section. Data are expressed as means ± SEM from 8 mice in each group at each time point. *p<0.05 when compared with respective controls.

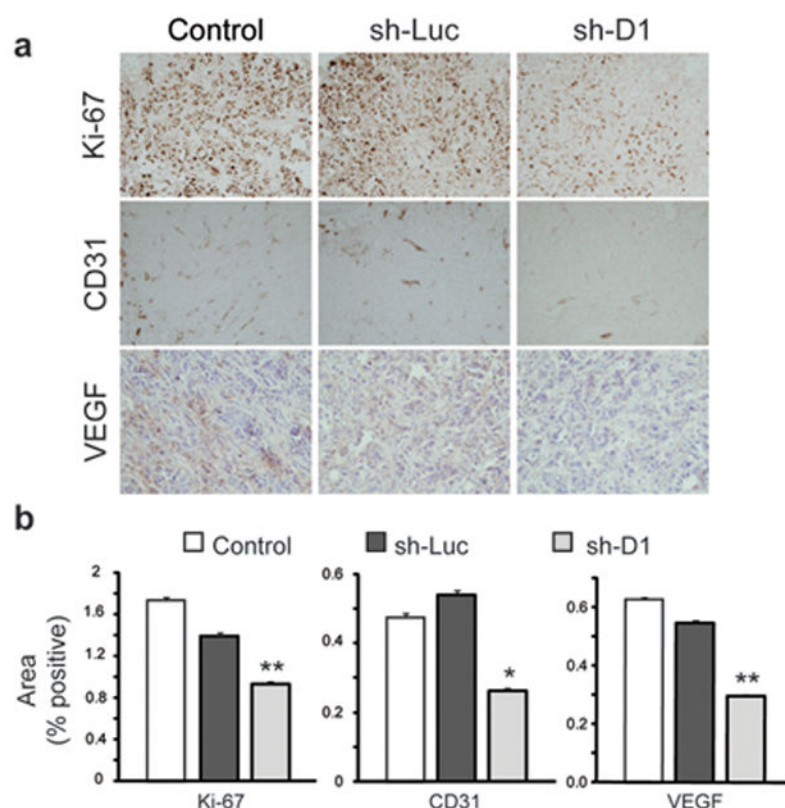


Figure 4. Effects of cyclin D1-directed shRNA on proliferation and angiogenesis in ASPC-1-derived tumors

(a) Immunohistochemical analysis. Representative immunostaining for Ki-67, CD31 and VEGF in ASPC-1-derived tumors that were injected *in vivo* with buffer (Control), a luciferase-directed shRNA-lentivirus (sh-Luc), or a cyclin D1-directed shRNA-lentivirus (sh-D1). Original magnification, x 20. **(b)** Quantitative morphometry for Ki-67, CD31, and VEGF immunoreactivity from the tumors shown in (a). Data are the means \pm SEM from three tumors/group, with 10 random fields analyzed for each tumor. * $P < 0.05$, ** $p < 0.01$ when compared with respective controls.

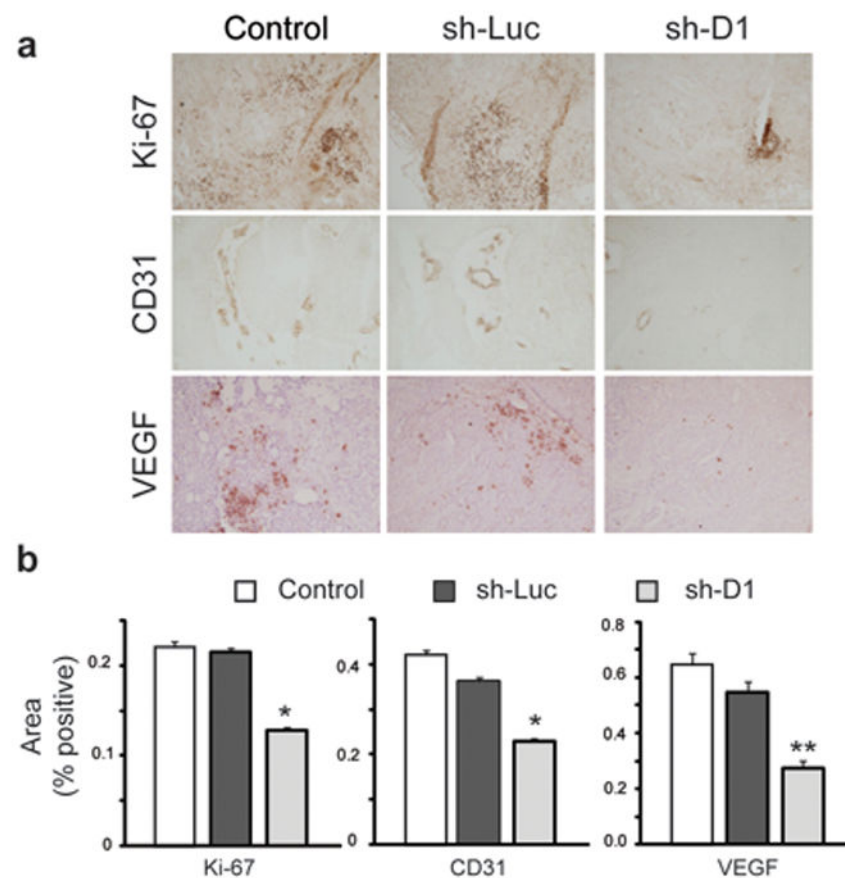


Figure 5. Effects of cyclin D1-directed shRNA on cell proliferation and angiogenesis in BxPC3-derived tumors

(a) Immunohistochemical analysis. Representative immunostaining for Ki-67, CD31 and VEGF in tumors BxPC3-derived tumors that were injected *in vivo* with buffer (Control), a luciferase-directed shRNA-lentivirus (sh-Luc), or a cyclin D1-directed shRNA-lentivirus (sh-D1). Original magnification, x 20. (b) Quantitative morphometry for Ki-67, CD31, and VEGF immunoreactivity. Data are the means \pm SEM from three tumors/cell line/group.

* $P < 0.05$, ** $p < 0.01$ when compared with respective controls.