Polo-like Kinase 1 Is Involved in Invasion through Extracellular Matrix

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Abstract

Polo-like kinase 1 (PLK1) has important functions in maintaining genome stability via its role in mitosis. Because PLK1 is up-regulated in many invasive carcinomas, we asked whether it may also play a role in acquisition of invasiveness, a crucial step in transition to malignancy. In a model of metaplastic basal-like breast carcinoma progression, we found that PLK1 expression is necessary but not sufficient to induce invasiveness through laminin-rich extracellular matrix. PLK1 mediates invasion via vimentin and β1 integrin, both of which are necessary. We observed that PLK1 phosphorylates vimentin on Ser82, which in turn regulates cell surface levels of β1 integrin. We found PLK1 to be also highly expressed in preinvasive *in situ* carcinomas of the breast. These results support a role for the involvement of PLK1 in the invasion process and point to this pathway as a potential therapeutic target for preinvasive and invasive breast carcinoma treatment. [Cancer Res 2007;67(23):11106–10]

Introduction

Polo-like kinase 1 (PLK1) is a member of the well-conserved family of polo-like kinases, which has four known members in humans: PLK1, PLK2, PLK3, and PLK4. Silencing of PLK1 via small interfering RNA (siRNA) induces apoptosis, interferes with mitosis (1, 2), inhibits centrosome amplification (3), and down-regulates response to DNA damage via BRCA2 phosphorylation (1). PLK1 mRNA level is transiently down-regulated in response to DNA damage, and this is dependent on BRCA1 and its downstream effectors, CHEK1 kinases (4). In addition to DNA damage and mitosis, PLK1 has been implicated in the Golgi checkpoint pathway that ensures proper segregation of this organelle during cell division (5).

Consistent with its known functions, PLK1 expression is regulated during cell cycle progression: levels are low in G_0, G_1, and S but begin to increase in G_2 and peak in M phase. In general, active proliferation has been correlated with high PLK1 levels and differentiation (induced by factors in culture) is correlated with low levels, whereas DNA damage acts as a transient down-regulator. In normal tissues, PLK1 is found only in actively proliferating tissues, such as placenta, and its expression increases in many invasive carcinomas, including those of the breast, ovarian, esophageal, head and neck, and skin (reviewed in ref. 6). Interestingly, PLK1 levels are also regulated by a direct interaction with the chaperone heat shock protein Hsp90, which has recently been linked to regulation of matrix metalloproteinase (MMP) function (7, 8).

Using the HMT-3522 cell line series, composed of the noninvasive S1 and S2, preinvasive S3-A, S3-B, and S3-C, and invasive T4-2 constituting a faithful model for the metaplastic basal-like breast cancer subtype (9, 10),[1] here we found a role for PLK1 in invasion, described a mechanism, and propose a therapeutic targeting strategy.

Materials and Methods

Cell culture. Cells were grown in tissue culture monolayers (two-dimensional) using Falcon tissue culture plastic or three-dimensional laminin-rich extracellular matrix (lrECM; Matrigel, BD Biosciences) in defined medium as described previously (11, 12). S2 and S3 cells were grown under the same conditions as T4-2.

Reverse transcription-PCR. Semiquantitative reverse transcription-PCR (RT-PCR) for PLK1 was performed using the following primers (5′-3′): aggctctgctcggatcga (forward) and tctctttcgccggtggag (reverse). After having determined linear range, conditions were as follows: 96°C for 3 min, 34× (96°C for 30 s, 58°C for 30 s, 72°C for 1 min), 72°C for 5 min.

Western blots. SDS-PAGE–based standard methods were used. Primary antibodies were the following: PLK1, rabbit polyclonal to peptide 8-21, PC382 (Chemicon) at 1:200 dilution; PLK2 (Novus) at 1:1,000 dilution; PLK4 (Novus) at 1:1,000 dilution; vimentin, rabbit polyclonal JM3634 (MBL International) at 1:100 dilution; and phosphorylated vimentin (Ser82), D095-3 (MBL International) at 1:500 dilution.

Invasion assay. Invasion through lrECM (Matrigel) was measured in Boyden chamber assays essentially as described (13). The number of invading cells (of 1×10^5 seeded) was determined after 48 h of incubation (unless indicated otherwise) in either regular growth medium, medium containing different concentrations of the GlaxoSmithKline compound, β1 function blocking antibody, and A2BII (Sierra BioSource), or medium containing 2-day conditioned medium from T4-2 cell cultures (for induction of invasion in S3-C cultures). For siRNA-treated T4-2 or S3-C cells, transfection of 30 to 150 nmol/L oligo with siPORT NeoFX (Ambion) was performed 24 h after plating cells. After 48 h in culture, siRNA-treated cells were trypsinized and seeded in Boyden chambers for invasion assays. siRNA oligos against PLK1 (3′ Alexa Fluor 488 labeled from Qiagen; DNA target sequence: cgacttcgtgttcgtggtg, described in ref. 1), vimentin (oligo 1: Ambion ID 138993; oligo 2: Ambion ID 138994; oligo 3: Ambion ID 138995), or scrambled control siRNA (Silencer Cy3 labeled; Ambion) were used.

Synchronization. T4-2 cells were kept in DMEM/F12 for 6 h to synchronize in G_1, after 2 days of siRNA inhibition by PLK1 or scrambled control. Cells were then plated over a thin layer of lrECM in chamber slides, in parallel to plating cells on a similar layer of lrECM for Boyden chamber invasion assays. The percentage of Ki67-positive cells per total 4′,6-diamidino-2-phenylindole–stained nuclei was evaluated at 3, 6, 12, 24, and 48 h after release into optimal growth medium. A minimum of 200 nuclei from three fields was counted for each replicate chamber slide.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling. Percentage apoptotic T4-2 cells after transfection with scrambled control or PLK1 siRNAs were detected using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay kit from Roche.

Cell surface expression. Live cells were immunostained in suspension before fixing with 2% paraformaldehyde. Primary antibodies were used at 1:10 dilution and secondary antibodies at 1:100 dilution. Fluorescence-activated cell sorting analysis was performed using EPICS XL-MCL data acquisition and display software on XL flow cytometry analyzers (University of California at Berkeley Flow Cytometry Facility). Gating of forward light scatter versus 90° light scatter allowed examination of intact cells only, and FITC fluorescence peak was evaluated for its median value and corrected using samples that had not been treated with primary antibody. Primary antibodies were as follows: β1 integrin, MAB1959 (Chemicon), and activated β1 integrin, HUTS-21 (BD PharMingen).

Tumorigenicity. We injected 2 million cells into the left and right fourth inguinal mammary glands of female BALB/c athymic nude mice (Simonsen Laboratories). To determine the effect of PLK1 down-regulation on tumorigenicity, PLK1 or scrambled control siRNAs were transfected into cells with 90% to 100% efficiency determined by using labeled siRNA oligos (Cy3 for scrambled and Alexa Fluor 488 for PLK1) and examining the percentage of cells containing the label by microscopy; cells were cultured for 4 days, injected into the fat pad, and allowed to form tumors for 5 weeks (minimum time needed for all T4-2 to form tumors).

Immunohistochemistry. Formalin-fixed, paraffin-embedded human breast tissue sections were obtained from the University of California at San Francisco (UCSF), Breast Specialized Program of Research Excellence, tissue core, or US Biomax, Inc. as 5-μm-thick serial sections. The samples from UCSF contained histologically normal, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) areas on the same section, as reported by the case pathologist and found in the archive records, and H&E sections were examined and confirmed by the UCSF tissue core staff. The tissue from US Biomax contained pure DCIS (Fmg020247B, Fmg020341B, Fmg020358B, and Fmg020585B). The paraffin was removed by incubation in xylene and graded alcohols. Tissues were blocked in 3% hydrogen peroxide in PBS. Antigen retrieval was performed by incubating in 0.01% prewarmed trypsin in PBS followed by microwaving in 10 mmol/L sodium citrate buffer. Tissues were blocked in 1.5% normal horse serum in PBS and incubated with 10 μg/mL of PLK1 antibody [anti-PLK1, human (rabbit); Calbiochem]. Slides were washed with PBS and incubated with biotinylated anti-rabbit antibody [1:200 dilution, biotinylated anti-mouse IgG/anti-rabbit IgG (H+L); Vector Laboratories] followed by streptavidin-horseradish peroxidase (Vectastain ABC kit, Elite; Vector Laboratories) and complete 3,3′-diaminobenzidine tetrahydrochloride (Sigma) medium. Slides were washed and counterstained with hematoxylin followed by dehydration in graded alcohols and xylene. Signal intensity in each cell was scored using a Zeiss Axioskop on a scale of 0 to 3. A minimum of 100 cells was counted for all existing distinct normal, DCIS, and IDC areas per case.

Results

Given that PLK1 levels are misregulated in invasive carcinomas, we asked whether it could be involved in acquisition of invasiveness. To determine whether there was a correlation between PLK1 expression levels and invasiveness in the HMT-3522 metaplastic breast carcinoma model (Fig. 1*A*), we examined PLK1 mRNA and protein levels in these cells, grown either in two-dimensional or three-dimensional lrECM cultures (Fig. 1*B*).

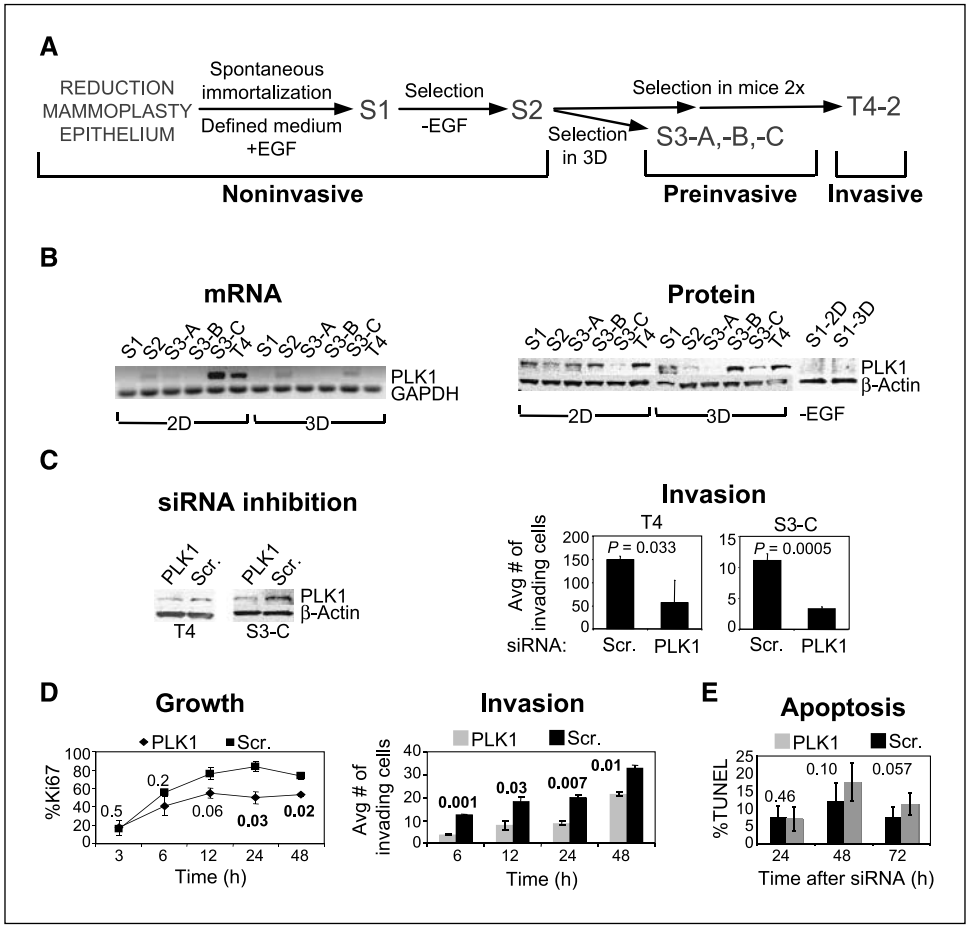

Figure 1. PLK1 expression is necessary but not sufficient for invasion. *A,* schematic presentation of the HMT-3522 model of metaplastic breast cancer progression (13). *B,* RT-PCR analysis for mRNA and Western blot for protein levels of PLK1. S1 cells in two-dimensional or three-dimensional lrECM were grown in the absence of epidermal growth factor (*EGF*) to completely growth arrest cells as a negative control for PLK1 signal. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. *C,* Western blot for PLK1 in cells transfected with siRNA to PLK1 versus scrambled control siRNA (*Scr.*); 44% and 46% reduction in T4-2 and S3-C cells, respectively. Invasion assay for T4-2 or S3-C cells (induced by T4-2 conditioned medium) after cells were transfected with PLK1 or scrambled control siRNA; three experiments, duplicate samples. *D,* the percentage of Ki67-positive T4-2 cells transfected with PLK1 (♦) or scrambled control (■) siRNAs at indicated time points after release from synchrony; invasion assay for these T4-2 cells. *E,* the percentage of TUNEL-positive T4-2 cells after transfection with the PLK1 or scrambled control siRNAs; two experiments, duplicate samples.

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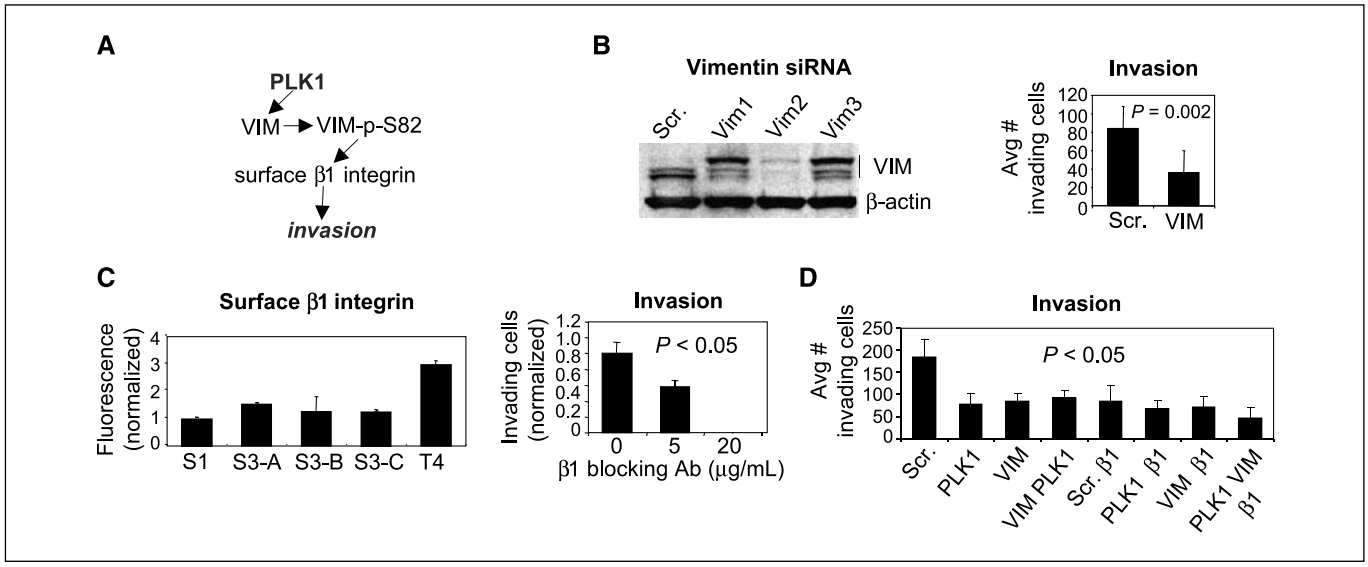

Figure 2. Vimentin (*VIM*) and β_1 integrin are necessary for PLK1-mediated invasion. *A,* the mechanism proposed. *B,* Western blot for vimentin in T4-2 cells transfected with the indicated siRNAs. Vim2 used for subsequent experiments. Invasion assay for T4-2 cells transfected with the indicated siRNAs. Four experiments, duplicate samples. *C,* cell surface expression of β1 integrin. Four experiments. Values normalized to S1. $P < 0.05$, between T4-2 and all other cell types. Invasion assay for T4-2 cells treated with the indicated amounts of β1 integrin blocking antibody A2BII. $P < 0.05$, compared with untreated control, six experiments. *D,* invasion assay for T4-2 cells treated with siRNAs against PLK1 or vimentin, or β1 blocking antibody, in combinations indicated; four experiments, duplicate samples. $P < 0.05$, compared with scrambled control siRNA.

Expression levels were different in S1, S2, S3-A, and S3-B cells and did not correlate directly with invasion, suggesting that PLK1 expression per se is not sufficient for invasion. Consistent with this, overexpression of PLK1 in S3-C cells had dominant-negative effects on growth, as had been observed also previously (14), and did not induce invasion (data not shown). To determine if PLK1 is necessary for invasion, we down-regulated PLK1 levels in T4-2 and in induced S3-C cells using a published siRNA against PLK1 (1), which did not down-regulate the levels of PLK homologues PLK2 or PLK4 (Supplementary Fig. S1). This specific down-regulation of PLK1 level significantly decreased invasion (Fig. 1*C*). In addition, a chemical inhibitor of PLK1 from GlaxoSmithKline induced a dose-dependent inhibition of invasion, confirming the siRNA results (data not shown for proprietary reasons dictated by GlaxoSmithKline).

To explore the relationship between the effect of PLK1 on growth and invasion, we synchronized cells before plating on lrECM in the chamber, determining both the proportion of cycling cells and the number of invading cells at 6, 12, 24, and 48 h (Fig. 1*D*). Ki67 staining at 3 h after release from synchronization showed that almost complete growth arrest was maintained for both PLK1 and control siRNA-treated T4-2 cells. At 6 and 12 h, there was no significant difference in percentage of Ki67-positive cells but the number of invading cells was significantly lower in the PLK1 siRNA-treated cells compared with control. The observation that the number of invading cells is down-regulated before the number of cycling cells is affected by PLK1 siRNA treatment allowed us to separate the effect of PLK1 on invasion from its effects on growth. In addition, using the proprietary GlaxoSmithKline inhibitor of PLK1 in T4-2 cells, we saw a reduction in invasion using two concentrations that were too low to affect growth (data not shown for proprietary reasons dictated by GlaxoSmithKline).

Because PLK1 siRNA has been shown to increase apoptosis in some cell types, we examined the effect of treating T4-2 cells with either PLK1 or scrambled control siRNA (Fig. 1*E*). Although there is a trend toward increased apoptosis in PLK1 siRNA-treated cells after 72 h, there was no significant increase in apoptosis during the time course of the invasion assay (48–72 h) to account for the observed effects of PLK1 down-regulation on invasion.

Based on reports that PLK1 phosphorylates vimentin on Ser82 (15), and that phosphorylation of vimentin by PKCε on NH_2-terminal serines (Ser4, Ser6, Ser7, Ser8, and Ser9 tested in combination) is important for retargeting of endocytosed β1 integrin to the cell surface in mouse embryonic fibroblasts (16), we postulated that PLK1 might function in cellular invasion by phosphorylating vimentin on Ser82, thereby affecting β1 integrin-mediated invasion through lrECM (Fig. 2*A*). Inhibition of vimentin by siRNA down-regulated invasion (Fig. 2*B*). β1 integrin was expressed at a higher level on the surface of T4-2 cells than in S1, S2, and S3s, and its inhibition resulted in a decrease of invasiveness in a dose-dependent manner (Fig. 2*C*). The effects of down-regulating PLK1, vimentin, and β_1 integrin function were not additive or synergistic in any combination (Fig. 2*D*), suggesting that the three proteins may function in the same invasion pathway. Knocking down PLK1 down-regulated the level of Ser82 phosphorylated vimentin (Fig. 3*A*), as well as decreasing the cell surface levels of β1 integrin (Fig. 3*C*), as did knocking down vimentin (Fig. 3*C*). Expressing mutant vimentin that contained a non-phosphorylatable Ser82 down-regulated invasion compared with the wild-type (WT) vimentin control, which had a higher level of phosphorylated Ser82 (Fig. 3*B*), as well as decreasing the total and activated β1 integrin levels on the cell surface (Fig. 3*D*).

To explore the relevance of this PLK1-mediated invasion mechanism to tumor phenotypes *in vivo*, we found that down-regulating PLK1 decreased the tumorigenicity of T4-2 cells in the mouse mammary fat pad (Fig. 4*A*). To ask whether PLK1 inhibition could potentially be used in treatment of preinvasive breast disease, we determined if *in situ* carcinomas expressed it. It was previously reported (6) that there was no detectable expression in normal tissues and that the invasive tumors displayed high

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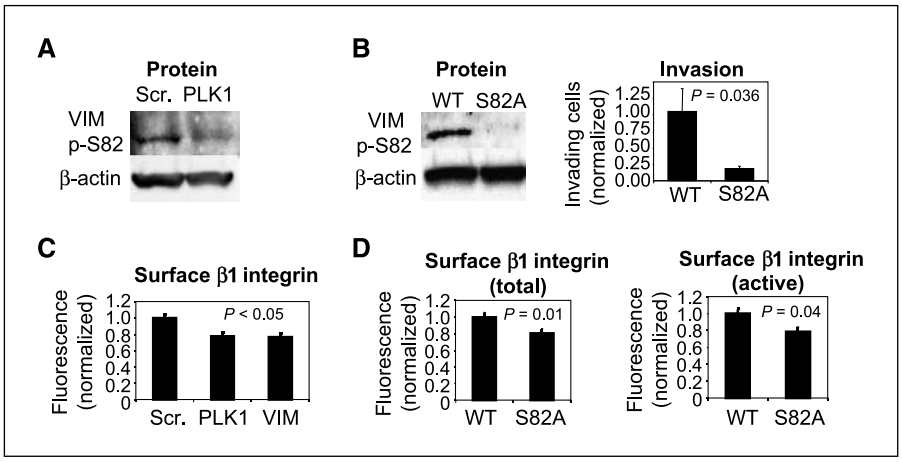

Figure 3. PLK1 affects invasion via phosphorylating vimentin and down-regulating cell surface β1 integrin. *A,* Western blot for Ser82 phosphorylated vimentin in T4-2 cells treated with the indicated siRNAs. *B,* Western blot for Ser82 phosphorylated vimentin in T4-2 cells infected with lentivirus expressing WT vimentin pVIM (*WT*) or mutated vimentin pVIM (*S82A*). Invasion assay for T4-2 samples infected with lentivirus expressing a WT vimentin pVIM (*WT*) or mutated vimentin pVIM (*S82A*). Normalized to WT values. $P = 0.036$, three experiments, triplicate samples. *C,* cell surface expression of β1 integrin on T4-2 cells treated with the indicated siRNAs. $P = 0.0007$ (PLK1-scrambled control siRNA) and 0.003 (vimentin-scrambled control siRNA); four experiments. *D,* cell surface β1 integrin levels (total and active, as indicated), normalized to T4-2 cells expressing WT vimentin; four experiments.

immunohistochemical signal. We confirmed these data: normal = 0.28 ± 0.15; invasive = 1.96 ± 0.06 (Fig. 4*B* and *D*). Interestingly, in the eight patient-matched biopsies we examined, *in situ* carcinoma lesions had higher levels of expression than invasive carcinoma regions on the same section: *in situ* = 2.64 ± 0.84; invasive = 1.90 ± 0.15 ($P = 1.57 \times 10^{-5}$; Fig. 4*C* and *D*). The high expression in DCIS was not simply because of being adjacent to IDC because in four pure DCIS samples examined we also observed high level of PLK1 expression (Supplementary Fig. S2). Given our finding above that a reduction in PLK1 reduces invasion significantly, these results may indicate the use of PLK1 inhibitory compounds in treatment of DCIS as well.

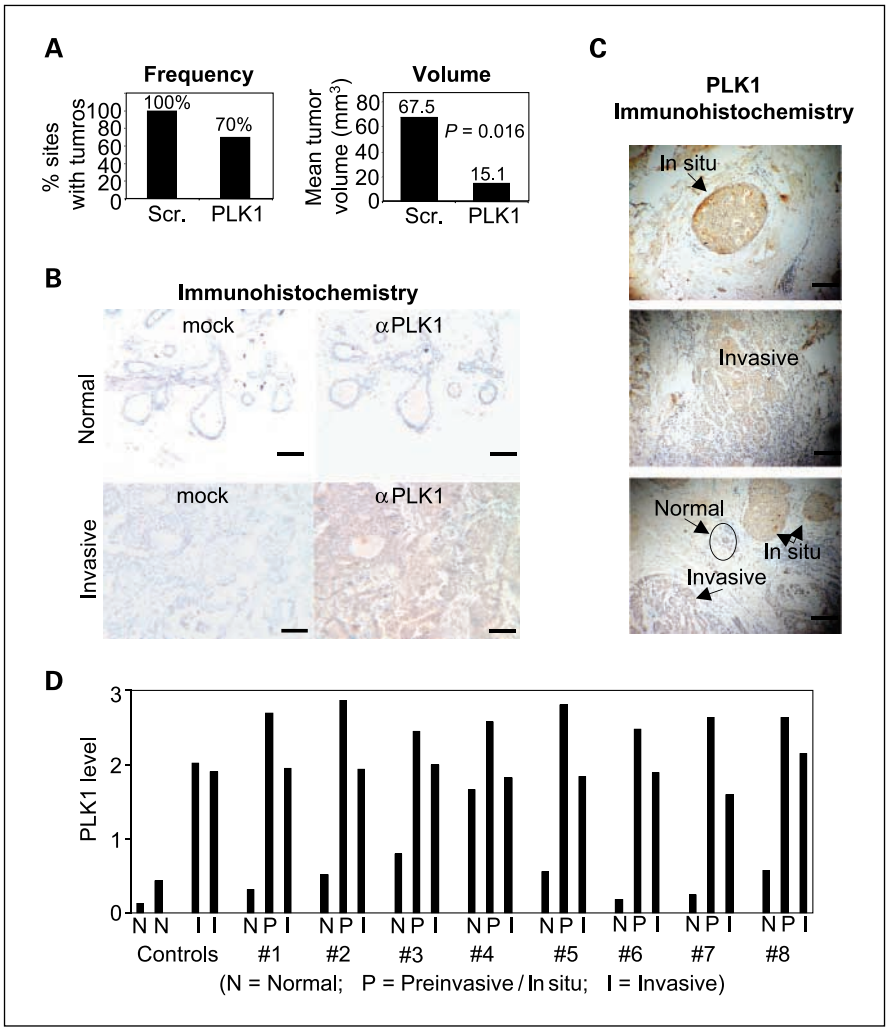

Figure 4. PLK1 *in vivo. A,* frequency of tumor formation and mean tumor volumes in fat pad. T4-2 transfected with siRNA against PLK1 ($n = 10$) versus scrambled control siRNA ($n = 5$). *B,* control experiment for immunohistochemical detection of PLK1, comparing mock antibody with PLK1 antibody-treated samples. *Bar,* 100 μm. *C,* example; PLK1 immunohistochemical signal in normal, *in situ*, and invasive samples from the same patient. *Bar,* 100 μm. *D,* PLK1 signal intensity. Two normal (*N*) and two invasive (*I*) cases as controls, compared with eight cases each containing areas of normal as well as *in situ*/preinvasive (*P*) and invasive carcinomas.

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Discussion

PLK1 is up-regulated in many invasive carcinomas and is important for maintaining genome stability via its functions in mitosis (17). Here, we found that PLK1 is involved also in acquisition of invasiveness in vimentin-expressing cells via regulating cell surface β_1 integrin levels (Figs. 2 and 3). Such acquired "moonlighting" functions have been described for several other proteins as well (18, 19). It is possible that the normal function of PLK1 in mediating intermediate filament-regulated events in cytokinesis manifests itself aberrantly when placed in the context of a malignant cell, resulting in a regulatory function for invasion. Although we showed a role for PLK1 in invasion via vimentin and β1 integrin, recent data on the involvement of the yeast polo-like kinase, CDC5, in cytokinesis via targeting and activation of RhoA at the cleavage furrow (20) suggest that actin filaments could potentially be involved in this regulation as well.

During mitosis, adherent cells first round up and decrease their attachment to the substratum, but after cytokinesis, they increase attachment and spread. The dynamics of attachment and detachment is critical for cells to go through mitosis (21). Consistent with this, we found the cell surface levels of β1 integrin to be regulated by PLK1 via phosphorylation of vimentin on Ser82 (Fig. 3). Importantly, however, we found that the effects of PLK1 on mitosis and invasion are separable (Fig. 2; GlaxoSmithKline compound data). Additional support for the separability of mitosis and invasion effects in this pathway comes from our observation that vimentin siRNAs, which down-regulate invasion, do not have any effects on growth (data not shown).

In addition to PLK1, Ku80, which is important in maintaining genome stability via double-strand break repair, is involved in invasion (19). Ku80 interacts with MMP9 on the cell surface, and its inhibition results in reduced activity of MMP9 and invasion. PLK1 down-regulation, however, does not affect MMP9 activity (data not shown), suggesting an MMP9-independent function. In addition, we found that the centromeric protein CENPA and the double-strand break repair protein XRCC3 were involved in invasion in Boyden chamber assays, whereas the M2 subunit of ribonucleotide reductase RRM2 was not (data not shown). We have now dubbed genes such as *PLK1, CENPA, XRCC3*, and *Ku80* as genomic instability and extracellular matrix invasion (GISEM) genes. Targeting the acquired or moonlighting invasion function in malignant cells without disrupting the ability of GISEM genes, such as *PLK1*, to maintain a stable genome in normal cells could contribute to the development of anticancer therapeutics with reduced toxicity.

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