Polo-like Kinase I is involved in Invasion through Extracellular Matrix

Aylin Rizki[#], Joni D. Mott, Mina J. Bissell[#]

[1]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

[#]Corresponding authors: Aylin Rizki and Mina J. Bissell, One Cyclotron Road, Mailstop 977R225A, Berkeley CA 94720. Tel: (510) 486-4365, Fax: (510) 486-5586, email: ARizki@lbl.gov, MJBissell@lbl.gov.

Running Title: PLK1 and Invasiveness in a Spontaneous Breast Cancer Model

Abstract

Polo-like kinase 1, PLK1, has important functions in maintaining genome stability and is involved in regulation of mitosis. 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 serine 82, which in turn regulates cell surface levels of β1 integrin. We found PLK1 to be also highly expressed in pre-invasive in situ carcinomas of the breast. These results support a role for the involvement of PLK1 in the invasion process and point to this athway as a potential therapeutic target for pre-invasive and invasive breast carcinoma treatment.

Introduction

PLK1, is a member of the well-conserved family of polo-like kinases which has four known members in humans, PLK1,2,3,4. Silencing of

PLK1 via 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 G0, G1, and S, but begin to increase in G2 and peak in M phase (reviewed in (6)). PLK1 is degraded via an APC dependent proteolysis pathway as cells exit mitosis. Interestingly, PLK1 phosphorylates APC, which in turn targets PLK1 for degradation. PLK1 levels are also regulated by a direct interaction with the chaperone heat shock protein Hsp90, which has recently been linked to regulation of MMP function (7, 8). Serum induces PLK1 mRNA levels in multiple human cell lines. In general, active proliferation has been correlated with high PLK1 levels, and differentiation (induced by factors in culture) with low levels, while 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 breast, ovarian, esophageal, head and neck, melanoma tumor specimens.

Using the HMT-3522 cell line series, comprised of the non-invasive S1 and S2, pre-invasive S3-A,-B,-C, and invasive T4-2 constituting a faithful model for the metaplastic basal-like breast cancer sub-type (9-11), here we found a role for PLK1 in invasion, described a mechanism, and propose a therapeutic strategy.

Materials and Methods

Cell culture. Cells were grown in tissue culture monolayers (2D) using Falcon^{TM} tissue culture plastic or 3DlrECM (Matrigel^{TM}, BD Biosciences) in defined medium as described previously (Petersen et al., 1992; Weaver et al., 1997). S2 and S3 cells were grown under the same conditions as T4-2.

RT-PCR. Semi-quantitative RT-PCR for PLK1 was performed using the following primers: forward; aggctctgctcggatcga, reverse; tctctttcgccggtggag, and conditions (after having determined linear range): 96^{o}C 3min, 34x(96^{o}C 30sec, 58^{o}C 30sec, 72^{o}C 1min), 72^{o}C 5min.

Western blots. SDS-PAGE based standard methods were used. Primary antibodies used were: PLK1 rabbit polyclonal to peptide 8-21, PC382(Chemicon), 1:200 dilution; vimentin, rabbit polyclonal, JM3634 (MBL international), 1:100 dilution; phospho vimentin (ser82) D095-3 (MBL International), 1:500 dilution.

Invasion assay. Invasion through lrECM (Matrigel) was measured in Boyden chamber assays, essentially as described (12). The number of invading cells (out of 1 X 10^5 seeded) was determined after 48 hrs of incubation (unless indicated otherwise) in either regular growth medium, in medium containing different concentrations the GSK compound, or of β1 function blocking antibody A2BII (Sierra Biosource), or medium containing 2-day conditioned medium from T4-2 cell cultures (for S3-C induction). For siRNA treated T4-2 or S3-C cells, transfection of 30-150nM oligo with siPORT NeoFX (Ambion) was performed 1 day after plating cells. After 2 days in culture, siRNA treated cells were trypsinized and seeded for Boyden chamber assays. siRNA oligos against PLK1 (3' Alexa 488 labeled from Qiagen, DNA target sequence: cgacttcgtgttcgtggtg, described in (1)), VIM (oligo1: Ambion ID 138993; oligo 2: Ambion ID 138994; oligo 3: Ambion ID 138995), or scrambled control siRNA (Ambion, Silencer^{TM}-Cy3 labeled) were used.

Synchronization. T4-2 cells were kept in DMEM/F12 for 6 hrs for synchronization, 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. Ki67 positive % was evaluated at 3, 6, 12, 24, and 48hrs after release into optimal growth medium, using DAPI staining to count total nuclei. A minimum of 200 nuclei from three fields were counted for each sample.

Cell surface expression. Live cells were immunostained in suspension prior to fixing with 2% paraformaldehyde. Primary antibodies were used at 1:10 dilution, and secondary antibodies at 1:100 dilution. FACS analysis was performed using EPICS XL-MCL data acquisition and display software on XL flow cytometry analyzers (UC Berkeley, Flow Cytometry Facility). Gating of FLS vs. LS allowed examination of intact cells only, FITC fluorescence peak was evaluated for its median value and was corrected using samples that had not been treated with primary antibody. Primary antibodies were: β1 integrin, MAB1959 (Chemicon); activated β1 integrin, HUTS-21 (BD Pharmigen);

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

downregulation on tumorigenicity, PLK1 or scrambled control siRNAs were transfected into cells at 90-100% efficiency, 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, Breast SPORE, tissue core, as 5 μm thick serial sections. The cases contained histologically normal, DCIS, and IDC areas on the same section, as reported by the case pathologist and found in the archive records. H&E sections were examined (by the UCSF tissue core staff) to confirm. 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% pre-warmed trypsin in PBS, followed by microwaving in 10mM sodium citrate buffer. Tissues were blocked in 1.5% normal horse serum in PBS and incubated with 10μg/ml of PLK1 antibody (Calbiochem, Anti-Plk1, Human (Rabbit), PC382). Slides were washed with PBS and incubated with biotinylated anti-rabbit antibody (1:200 dilution, Vector Laboratories, Biotinylated anti-mouse IgG / anti-rabbit IgG (H+L), BA-1400), followed by streptavidin-HRP (Vector Laboratories, Vectastain ABC kit, Elite PK-6100) , and complete DAB (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 were counted for all existing distinct normal, DCIS and IDC areas for each case.

Results

To determine whether there was a correlation between PLK1 expression levels and acquisition of invasiveness in the HMT-3522 metaplastic breast carcinoma model (Figure 1A), we examined PLK1 mRNA and protein levels in these cells, grown either in 2D or 3DlrECM cultures (figure 1B). Expression levels varied 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, over expression of PLK1 in S3-C cells had dominant negative effects on growth, as had also been previously observed (13), and was not sufficient to induce invasiveness (data not shown). To determine if PLK1 is necessary for invasion, we down regulated PLK1 levels in T4-2 and induced S3-C cells using siRNA (1), and found that this significantly decreased invasion (Figure 1C). We also found that a chemical inhibitor of PLK1 from GlaxoSmithKlein (GSK) induced a

dose-dependent inhibition of invasion, confirming the siRNA results (data not shown for proprietary reasons dictated by GSK).

To explore the relationship between the effect of PLK1 on growth and invasion, we synchronized cells prior to 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 hrs (Figure 1D). Ki67 staining at 3 hrs 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 hrs, there was no significant difference in Ki67 positivity but the number of invading cells was significantly lower in the PLK1 siRNA-treated cells compared to 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 GSK inhibitor of PLK1 in T4-2 cells, we saw a reduction in invasion using two low concentrations that did not affect growth (data not shown for proprietary reasons dictated by GSK).

Based on reports that PLK1 phosphorylates vimentin (VIM) on ser82 (14), and phosphorylation of vimentin by PKCε on N-terminal serines (ser4,6,7,8,9 tested in combination) is important for retargeting of

endocytosed β1 integrin to the cell surface in mouse embryonic fibroblasts (15), we postulated and tested the hypothesis that PLK1 could function in cell invasiveness by phosphorylating vimentin on serine 82 thereby affecting β1 integrin-mediated invasion through lrECM (Figure 2A). siRNA inhibition of vimentin down regulated invasion (Figure 2B); 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 (Figure 2C). Effects of downregulating PLK1, vimentin, and β1 integrin function were not additive or synergistic in any combination (Figure 2D) suggesting that the three proteins function in the same invasion pathway. Knocking down PLK1 down regulated the level of ser82 phosphorylated vimentin (Figure 3A), as well as decreasing the cell surface levels of β1 integrin (Figure 3C), as did knocking down vimentin (Figure 3C). Expressing mutant vimentin which contained a non-phosphorylatable ser82 down regulated invasion compared to the wildtype vimentin control which had a higher level of phosphorylated ser82 (Figure 3B), as well as decreasing the total and activated β1 integrin levels on the cell surface (Figure 3D).

To explore the relevance of this PLK1-mediated invasion mechanism to tumor phenotypes *in vivo*, we found that downregulating PLK1 decreased the tumorigenicity of T4-2 cells in the mouse mammary fat

pad (Figure 4A).To ask if PLK1 inhibition could potentially be used in treatment of pre-invasive breast disease, as well as malignant lesions in human breast cancer which had previously been reported to express high levels of PLK1 protein, we determined if in situ carcinomas expressed it, after confirming the published findings (6) that there was no detectable expression in normal tissues and that the invasive tumors displayed high immunohistochemical signal: normal = 0.28 +/- 0.15; invasive = 1.96 +/- 0.06 (Figure 4B,D). In the 8 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-value = 1.57×10^{-5} (Figure 4C,D).

Discussion

PLK1 has been shown to be up regulated in many malignant cancers, and is important for maintaining genome stability via its functions in mitosis (16). Here, we found that PLK1 is also necessary (but not sufficient) in acquisition of invasiveness in vimentin expressing cells via regulating cell surface β1 integrin levels (Figure 2-3). Such acquired "moonlighting" functions have been attributed to a number of other proteins as well (17, 18). 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 demonstrated a role for PLK1 in invasion via vimentin and β1 integrin, recent data for the involvement of the yeast polo-like kinase CDC5 in cytokinesis via targeting and activation of RhoA at the cleavage furrow (19) suggest that actin filaments could potentially be involved in this regulation.

During mitosis, adherent cells first round up and decrease their attachment to the substrate, but after cytokinesis they increase attachment and spread. The dynamics of attachment and detachment is critical for cells to go through mitosis (20). Consistent with this, we found the cell surface levels of β1 integrin to be regulated by PLK1 via phosphorylation of vimentin on serine 82 (Figure 3). However, in this model, we also found the effects of PLK1 on mitosis and invasion to be separable (Figure 2, and GSK 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, another protein involved in maintaining genome stability, the double-strand break repair protein Ku80, has been shown to be involved in invasion (18). Ku80 interacts with MMP9 on the cell surface, and its inhibition results in reduced invasion. PLK1 down regulation 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 these genes *Genomic Instability and Extracellular Matrix Invasion (GISEM)* genes. Targeting the acquired or "moonlighting" invasion function without disrupting the ability of GISEM genes such as PLK1 to maintain a stable genome in normal cells could constitute new anti-cancer therapies with reduced toxicity.

Acknowledgements

We thank Wen-Lin Kuo for technical assistance and/or advice, the UCSF tissue utilization committee, and especially Karen Chew, for providing human breast tissue sections and advice. This work was supported by CBCRP Postdoctoral Fellowship Award 8FB0184 to A.R; DOD BCRP Predoctoral Fellowship Award DAMD170310421 to G.I.R.; NIH grant CA88858 support for J.D.M; NIH grant CA58207 to J.W.G.; US DOE OBER grant DEAC0376SF00098, US DOE OBER Distingushed Scientist Fellowship, DOD BCRP Innovator Award DAMD170210438; and NIH grant CA64786 to M.J.B.

Figure legends

Figure 1. PLK1 is necessary but not sufficient for invasion

A. The HMT-3522 model of metaplastic breast cancer progression (9). **B.** RT-PCR analysis for mRNA and Western blot for protein levels of PLK1. S1 cells in 2D or 3DlrECM were grown in the absence of EGF in order to completely growth arrest cells as a negative control for PLK1 signal. **C.** Western blot for PLK1 in cells transfected with siRNA to PLK1 vs. Scr.; 44% and 46% reduction in T4-2 and S3-C cells, respectively; and invasion assay for T4-2 or S3-C cells (induced by T4-2 CM), after cells were transfected with PLK1 vs. Scr. siRNA; three experiments, duplicate samples. **D.** %Ki67 positive T4-2 cells transfected with PLK1(▪) or (▪) Scr. siRNAs, at indicated time points after release from synchrony; and invasion assay for theseT4-2 cells.

Figure 2. Vimentin, and β1 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; and 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; and invasion assay for T4-2 cells treated with the indicated amounts of β1 integrin blocking antibody A2BII; $p<0.05$ compared to 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 to Scr.

Figure 3. PLK1 affects invasion via phosphorylating Vimentin and downregulating cell-surface β1 integrin

A. Western blot for serine 82 phosphorylated vimentin in T4-2 cells treated with the indicated siRNAs. **B.** Western blot for serine 82 phosphorylated vimentin in T4-2 cells infected with lentivirus expressing wildype vimentin pVIM (WT) or mutated vimentin pVIM (S82A); and 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-value (PLK1-Scr.) = 0.0007, p-value (VIM-Scr.) = 0.003; four experiments. **D.** Cell surface β1 integrin levels (total and active, as indicated), normalized to T4-2 cells expressing WT vimentin; four experiments.

Figure 7. PLK1 *in vivo*

A. Frequency of tumor formation and mean tumor volumes in fat pad; T4-2 transfected with siRNA against PLK1 (n=10) vs. Scr. (n=5). **B.** Control experiment for immunohistochemical detection of PLK1, comparing mock antibody to PLK1 antibody treated samples, scale bar 100μm. **C.** Example; PLK1 immunohistochemical signal in normal, in situ, and invasive samples, from the same patient, scale bar 100μm. **D.** PLK1 signal intensity; 2 normal (N) and 2 invasive (I) cases as controls, compared to 8 cases each containing areas of normal (N), as well as in situ / pre-invasive (P) and invasive carcinomas (I).

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Figure 1.

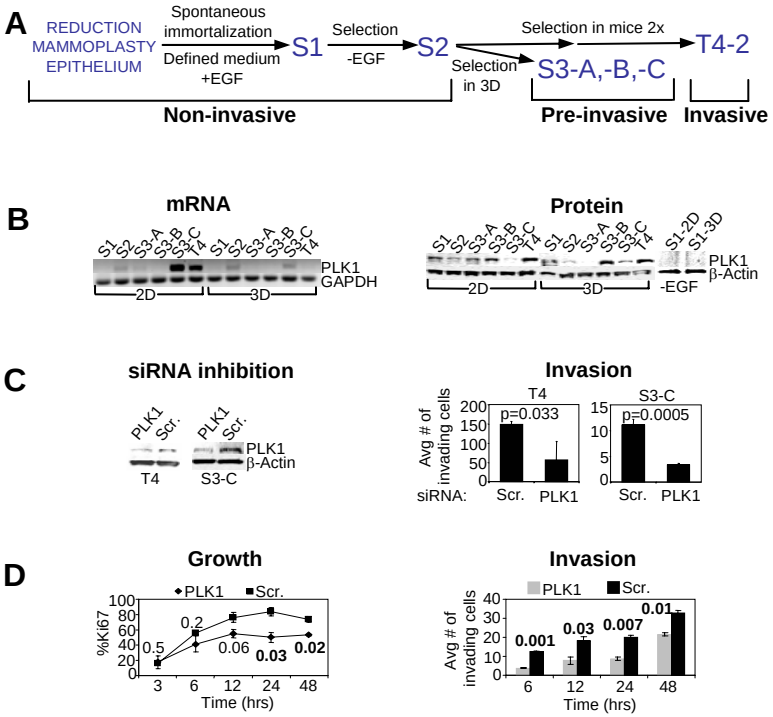
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Figure 2.

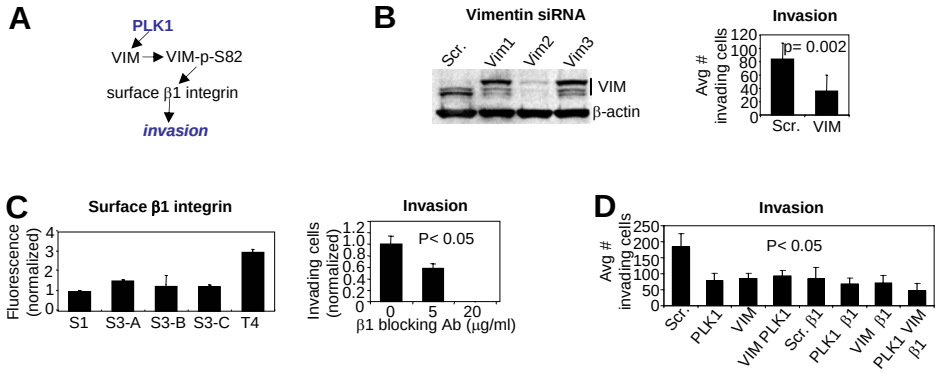
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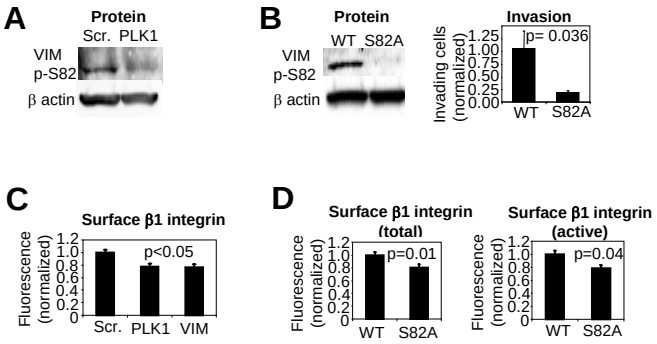
Figure 3.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1.0
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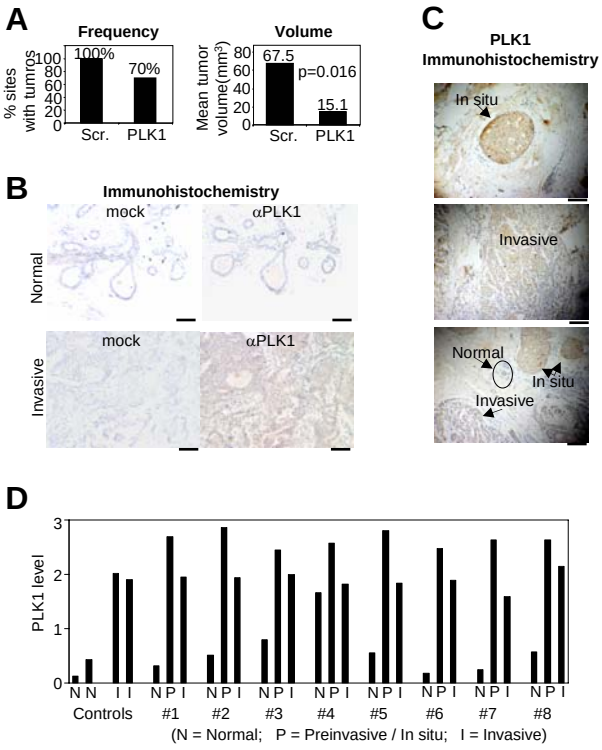
Figure 4.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αPLK1
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(N = Normal; P = Preinvasive / In situ; I = Invasive)