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

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

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.

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

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

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

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 DE-AC02-05CH11231, US DOE OBER Distingushed Scientist Fellowship, DOD BCRP Innovator Award DAMD170210438; and NIH grant CA64786 to M.J.B.

$p<0.05$ 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*

References

1. Lee, M., Daniels, M. J., and Venkitaraman, A. R. Phosphorylation of BRCA2 by the Polo-like kinase Plk1 is regulated by DNA damage and mitotic progression. Oncogene, *23:* 865-872, 2004.

2. Reagan-Shaw, S. and Ahmad, N. Silencing of polo-like kinase (Plk) 1 via siRNA causes induction of apoptosis and impairment of mitosis machinery in human prostate cancer cells: implications for the treatment of prostate cancer. Faseb J, *19:* 611-613, 2005.

3. Liu, X. and Erikson, R. L. Activation of Cdc2/cyclin B and inhibition of centrosome amplification in cells depleted of Plk1 by siRNA. Proc Natl Acad Sci U S A, *99:* 8672-8676, 2002.

4. Ree, A. H., Bratland, A., Nome, R. V., Stokke, T., and Fodstad, O. Repression of mRNA for the PLK cell cycle gene after DNA damage requires BRCA1. Oncogene, *22:* 8952-8955, 2003.

5. Preisinger, C., Korner, R., Wind, M., Lehmann, W. D., Kopajtich, R., and Barr, F. A. Plk1 docking to GRASP65 phosphorylated by Cdk1 suggests a mechanism for Golgi checkpoint signalling. Embo J, *24:* 753-765, 2005.

6. Winkles, J. A. and Alberts, G. F. Differential regulation of polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues. Oncogene, *24:* 260-266, 2005.

7. Picard, D. Hsp90 invades the outside. Nat Cell Biol, *6:* 479-480, 2004.

8. Eustace, B. K., Sakurai, T., Stewart, J. K., Yimlamai, D., Unger, C., Zehetmeier, C., Lain, B., Torella, C., Henning, S. W., Beste, G., Scroggins, B. T., Neckers, L., Ilag, L. L., and Jay, D. G. Functional proteomic screens reveal an essential extracellular role

15. Ivaska, J., Vuoriluoto, K., Huovinen, T., Izawa, I., Inagaki, M., and Parker, P. J. PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility. Embo J, *24:* 3834-3845, 2005.

16. Eckerdt, F., Yuan, J., and Strebhardt, K. Polo-like kinases and oncogenesis. Oncogene, *24:* 267-276, 2005.

17. Jeffery, C. J. Moonlighting proteins: old proteins learning new tricks. Trends Genet, *19:* 415-417, 2003.

18. Muller, C., Paupert, J., Monferran, S., and Salles, B. The double life of the Ku protein: facing the DNA breaks and the extracellular environment. Cell Cycle, *4:* 438-441, 2005.

19. Yoshida, S., Kono, K., Lowery, D. M., Bartolini, S., Yaffe, M. B., Ohya, Y., and Pellman, D. Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. Science, *313:* 108-111, 2006.

20. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A. Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. Oncogene, *19:* 5606-5613, 2000.

Figure 2.

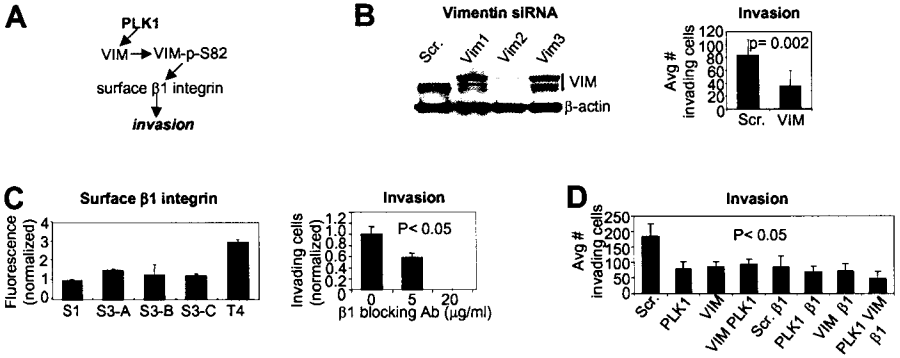
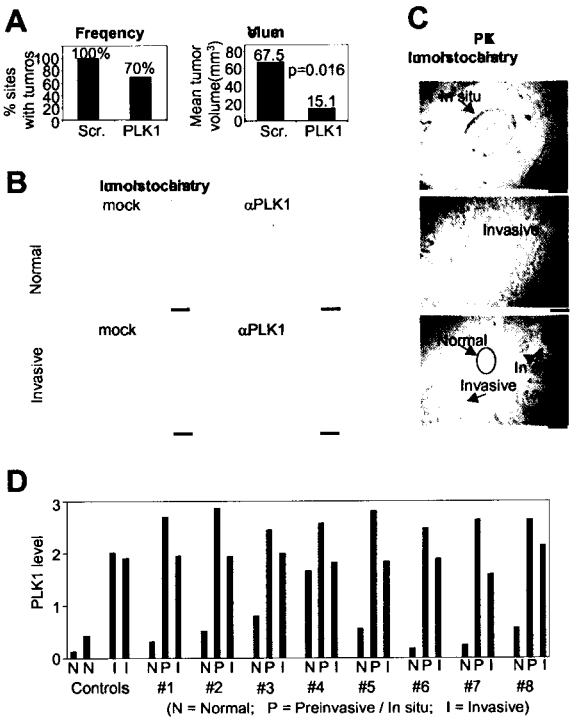

A
PLK1
VIM → VIM-p-S82
surface β1 integrin
invasion
B
Vimentin siRNA
Scr.
Vim1
Vim2
Vim3
VIM
β-actin
Invasion
Avg # invading cells
p= 0.002
Scr.
ViM
C
Surface β1 integrin
Fluorescence (normalized)
S1
S3-A
S3-B
S3-C
T4
Invasion
Invading cells (normalized)
P< 0.05
β1 blocking Ab (μg/ml)
D
Invasion
Avg # invading cells
P< 0.05
Scr.
PLK1
VIM
VIM PLK1
Scr. β1
PLK1 β1
VIM β1
PLK1 VIM β1

Figure 4


A
Freqency
100%
70%
% sites with tumros
Scr.
PLK1
Mean tumor volume(mm³)
67.5
p=0.016
15.1
Scr.
PLK1
B
Immunohistochemistry
mock
αPLK1
Normal
mock
αPLK1
Invasive
C
PLK
Immunohistochemistry
In situ
Invasive
Normal
Invasive
D
PLK1 level
0
1
2
3
N N I I N P I N P I N P I N P I N P I N P I N P I N P I
Controls #1 #2 #3 #4 #5 #6 #7 #8
(N = Normal; P = Preinvasive / In situ; I = Invasive)