Small interfering RNA library screen of human kinases and phosphatases identifies polo-like kinase 1 as a promising new target for the treatment of pediatric rhabdomyosarcomas

Kaiji Hu,1 Cathy Lee,1 Dexin Qiu,2 Abbas Fotovati,1 Alastair Davies,1 Samah Abu-Ali,1 Daniel Wai,3 Elizabeth R. Lawlor,3 Timothy J. Triche,3 Catherine J. Pallen,2 and Sandra E. Dunn1

1Laboratory for Oncogenomic Research, Departments of Pediatrics, Experimental Medicine, and Medical Genetics, and 2Cell Phosphosignaling Laboratory, Departments of Pediatrics, Pathology and Laboratory Medicine, and Experimental Medicine, Child and Family Research Institute, University of British Columbia, Vancouver, British Columbia, Canada; and 3Department of Pathology, Keck School of Medicine of the University of Southern California, Los Angeles, California

Abstract
Rhabdomyosarcoma, consisting of alveolar (aRMS) and embryonal (eRMS) subtypes, is the most common type of sarcoma in children. Currently, there are no targeted drug therapies available for rhabdomyosarcoma. In searching for new molecular therapeutic targets, we carried out genome-wide small interfering RNA (siRNA) library screens targeting human phosphatases (n = 206) and kinases (n = 691) initially against an aRMS cell line, RH30. Sixteen phosphatases and 50 kinases were identified based on growth inhibition after 72 hours. Inhibiting polo-like kinase 1 (PLK1) had the most remarkable impact on growth inhibition (~80%) and apoptosis on all three rhabdomyosarcoma cell lines tested, namely, RH30, CW9019 (aRMS), and RD (eRMS), whereas there was no effect on normal muscle cells. The loss of PLK1 expression and subsequent growth inhibition correlated with decreased p-CDC25C and Cyclin B1. Increased expression of WEE1 was also noted. The induction of apoptosis after PLK1 silencing was confirmed by increased p-H2AX, propidium iodide uptake, and chromatin condensation, as well as caspase-3 and poly(ADP-ribose) polymerase cleavage. Pediatric Ewing’s sarcoma (TC-32), neuroblastoma (IMR32 and KCNR), and glioblastoma (SF188) models were also highly sensitive to PLK1 inhibition. Finally, based on cDNA microarray analyses, PLK1 mRNA was overexpressed (>1.5 fold) in 10 of 10 rhabdomyosarcoma cell lines and in 47% and 51% of primary aRMS (17 of 36 samples) and eRMS (21 of 41 samples) tumors, respectively, compared with normal muscles. Similarly, pediatric Ewing’s sarcoma, neuroblastoma, and osteosarcoma tumors expressed high PLK1. We conclude that PLK1 could be a promising therapeutic target for the treatment of a wide range of pediatric solid tumors including rhabdomyosarcoma. [Mol Cancer Ther 2009;8(11):3024–35]

Introduction
Rhabdomyosarcoma is the most common soft-tissue sarcoma of children and adolescents. The majority (66%) of cases of rhabdomyosarcoma are diagnosed in children <6 years of age (1). This disease is thought to arise from primitive mesenchymal progenitors that have undergone a limited program of myogenic differentiation (2). Rhabdomyosarcoma consists of a highly heterogeneous family of tumors showing varying degrees of skeletal muscle differentiation (3). Embryonal rhabdomyosarcoma (eRMS) and the morphologic spindle/botryoid variants are associated with intermediate and superior patient prognosis, respectively, whereas alveolar rhabdomyosarcoma (aRMS) is more aggressive, with a high frequency of metastasis at the time of initial diagnosis (4). Current treatment for rhabdomyosarcoma includes chemotherapy, radiation, and surgery. The “gold standard” chemotherapeutic agents vincristine, actinomycin D, and cyclophosphamide are commonly prescribed to rhabdomyosarcoma patients (1). Chemoresistance is fairly common as are treatment-related side effects (1, 5). This is coupled with the fact that the present cure rate for children with metastatic rhabdomyosarcoma is still only 20% to 30% (6, 7). Unlike advanced treatment strategies for other types of malignancies, there are no targeted drug therapies available for rhabdomyosarcoma that could potentially improve overall cure rates and reduce morbidity. Thus, identifying new molecular targets of the disease is necessary.

Loss of heterozygosity on the short arm of chromosome 11 (1p15.5) characterizes eRMS (8). In contrast, aRMS harbors the reciprocal chromosomal translocations t(2;13)(q35; q14) or t(1;13)(p36;q14), generating a chimeric fusion gene...
involving the PAX3 gene (chromosome 2) or PAX7 (chromosome 1) and FKHR (chromosome 13), a member of the fork-head family. The resulting gene fusions encode PAX3-FKHR and PAX7-FKHR proteins that combine trans-criptional domains from corresponding wild-type proteins and are more potent transcription factors. These proteins induce cell transformation and inhibit myogenic differentiation and apoptosis, thereby enhancing oncogenic activity. Recently, several gene expression studies of primary rhabdomyosarcoma tumors have provided new information about the pathways involved in rhabdomyosarcoma (6, 12–19). New evidence indicates that aRMS can be experimentally induced by expressing the PAX/FKHR fusion gene in mesenchymal stem cells following the introduction of activating RAS mutation (20). Despite new knowledge and the belief that rhabdomyosarcoma arises from disrupted proliferation and differentiation of skeletal muscle progenitor cells, the mechanisms of growth control of rhabdomyosarcoma are not fully understood.

Kinases and phosphatases control the reversible processes of phosphorylation and are deregulated in many diseases, such as cancer. A recent study of genome-wide small interfering RNA (siRNA) libraries against the HeLa cervical carcinoma cell line has shown that a variety of phosphatases and kinases are critical in cancer cell survival. Kinase inhibitors targeting PAX3-FKHR, IGF-IR, CDK4/6, and EGFR have also shown potent antitumorigenic activity on rhabdomyosarcoma under in vitro and in vivo conditions (5, 7, 22–24). It is reported that the phosphorylation levels of receptors and nonreceptor tyrosine kinases, as well as protein kinase C, are elevated in rhabdomyosarcoma tumors and therefore have high therapeutic potential. These studies indicate that interfering with key signal transduction pathways may lead to improved therapies for rhabdomyosarcoma. However, genome-wide screens have not been reported to date.

In this study, we screened two siRNA libraries of 897 human phosphatases and kinases against an aRMS cell line, RH30 (SJCRH30), with the goal of finding novel therapeutic targets for this particular type of cancer.

**Materials and Methods**

**Cell Lines**

Rhabdomyosarcoma cell lines RH30, CW9019, and RD, and a mouse muscle cell line, C2C12, were cultured in DMEM purchased from Invitrogen. A human pediatric glioblastoma multiforme cell line, SF188, was cultured in MEM/Earle’s Balanced Salts medium from HyClone. An Ewing’s sarcoma, TC-32, and two neuroblastoma cell lines, IMR32 and KCNR, were maintained in RPMI 1640 medium from Invitrogen. All media contained 10% fetal bovine serum except for TC-32, IMR32, and KCNR cells that were grown in medium with 15% fetal bovine serum. The cell lines were maintained at 5% CO2 at 37°C and subcultured twice weekly during the experimental period.

**Phosphatase and Kinase siRNA Libraries**

The siRNA libraries (V2.0) of 206 phosphatases and 691 kinases were purchased from Qiagen. There were two different sequences of siRNAs targeting each of the genes in the libraries. The siRNA samples were supplied in 96-well plates. They were diluted to working stocks at 2 μmol/L upon arrival following the manufacturer’s instructions, and were stored at −20°C until use.

**siRNA Library Screen and High Content Screening Analysis**

RH30 cells were seeded (5,000/well) into each well of 96-well plates (Becton Dickinson) overnight. The cells were then transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions. The final concentration of siRNA was 5 nmol/L in 120 μL medium per well. The assay plates were incubated at 37°C with 5% CO2 for 72 h. Forty minutes before the end of siRNA treatment, nuclear dyes, Hoechst 33342, and propidium iodide (Sigma-Aldrich) were added to each well of the 96-well plates to give a final concentration of 1 μg/mL of each dye, and the plates were incubated as before. The cells were washed gently with PBS three times before the cells were fixed in 2% paraformaldehyde. The plates were kept at 4°C in the dark before analysis on the ArrayScan HCS system (Thermo Fisher Scientific). Twenty focus fields per cell well were scanned and analyzed. The screen was repeated at least once to confirm the activity of siRNAs. Cells treated with Lipofectamine RNAiMAX alone without siRNA served as controls. Additionally, scrambled siRNAs and green fluorescent protein siRNAs were included in the libraries, and served as internal references in each assay plate. Apoptosis was identified by nuclear morphology and dye intensity by the ArrayScan HCS system (26). Growth inhibition was calculated as a percentage of the control. To focus on the phosphatases and kinases with the most significant effect on cell growth, only those siRNAs that were active for both sequences and showed a minimum of 30% inhibition compared with control were considered to be active in the screen.

**Effects of Silencing the Selected Phosphatases and Kinases in Different Rhabdomyosarcoma Cell Lines**

To evaluate whether the active phosphatases and kinases identified in the primary screen are similarly active in different rhabdomyosarcoma cell types, 12 phosphatases and 16 kinases were silenced in two additional rhabdomyosarcoma cell lines, CW9019 (aRMS) and RD (eRMS). The experimental methods were the same as described above for the library screen.

**Analysis of the Active Genes by IPA**

To explore the possible links or interactions among the active phosphatases and kinases identified in the siRNA library screen, IPA software by Ingenuity Systems was employed to further analyze these genes and to group them into functional categories and cell signaling pathways.

**Effect of PLK1 Knocking Down on Pediatric Cancer Cell Lines In vitro**

To test the effect of silencing PLK1 on cell growth, the RH30, CW9019, RD, SF188, and C2C12 cell lines were cultured and transfected with PLK1 siRNA as described above for the library screen with the addition of six replicates for each treatment.

**Immunofluorescent Assays and Immunoblotting**

To directly visualize the expression of PLK1 in cells, RH30, CW9019, RD, and SF188 cells were seeded at 1.0 × 10⁵ cells on glass cover slips, washed with PBS, fixed with...
2% formaldehyde for 20 min, and rinsed twice with PBS. The slides were then incubated with PBS containing 0.1% saponin (Sigma-Aldrich) for 30 min. Next, the cover slips were washed with PBS, and incubated with either rabbit anti-PLK1 (8–21) antibody (1:100; Calbiochem) or (for double-staining) a mixture of mouse anti-PLK1 antibody (1:100; Sigma-Aldrich) with rabbit antibody against either p-CDC25C Ser198 (1:100; Cell Signaling Technology), cyclin B1 (M-20), or WEE1 (C-20) (1:100; Santa Cruz Biotechnology) dissolved in buffer containing 10% bovine serum albumin and 2% goat serum for 1 h at room temperature in a humidified container. After washing three times with PBS, the slides were incubated with Alexa 488 antirabbit antibody or a mixture of Alexa 546 antimouse antibody and Alexa 488 antirabbit antibody (for double-staining) for 1 h, washed three times, and then mounted with Vectashield mounting medium from Vector Laboratories. Hoechst 33342 dye was used for nuclear staining.

The silencing efficacy of PLK1 siRNA on protein expression was tested using standard SDS-PAGE methods (27) on a panel of pediatric cancer cell lines, including RH30, CW9019, RD, SF188, TC-32 (Ewing’s sarcoma), IMR32 and KCNR (neuroblastoma), and C2C12 (mouse myoblast). Primary antibodies used for the studies and their dilutions were as follows: anti-PLK1 (1:5,000; Sigma-Aldrich), anti-caspase 3 (cleaved), anti–poly(ADP-ribose) polymerase (cleaved), and anti–pan-actin (1:1,000; Cell Signaling Technology). Apoptosis of the cells was simultaneously assessed by probing with anti–p-H2AX S139 antibody (1:1,000) from Abcam. Apoptosis after PLK1 siRNA treatment of RH30, CW9019, RD, and SF188 cells was also analyzed quantitatively on the HCS system using propidium iodide and p-H2AX S139 as indicators as described (26).

PLK1 mRNA Expression in Rhabdomyosarcoma Cell Lines and Primary Tumors

Rhabdomyosarcoma cell lines used for microarray analysis included five eRMS cell lines (Birch, RD, TTC-442, TTC-516, and TTC-1318), one aRMS fusion-negative cell line (RH18), and four aRMS FAX3-FKHR fusion-positive cell lines (HR, JR-C, RH28, and RH30). All rhabdomyosarcoma cell lines used for microarray analysis included five eRMS cell lines (Birch, RD, TTC-442, TTC-516, and TTC-1318), one aRMS fusion-negative cell line (RH18), and four aRMS FAX3-FKHR fusion-positive cell lines (HR, JR-C, RH28, and RH30).
Table 2. Active kinases identified in the siRNA library screen and their status in primary tumors

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Symbol</th>
<th>Gene description</th>
<th>Growth inhibition, % (sequence C)</th>
<th>Growth inhibition, % (sequence D)</th>
<th>Gene expression status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell cycle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_000217</td>
<td>AURKB</td>
<td>Aurora kinase B</td>
<td>56.1</td>
<td>53.7</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_001204</td>
<td>BMPR2</td>
<td>Bone morphogenetic protein receptor, type II (serine/threonine kinase)</td>
<td>43.2</td>
<td>35.4</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_001743</td>
<td>CALM2</td>
<td>Calmodulin 2 (phosphorylase kinase, δ)</td>
<td>32.7</td>
<td>39.4</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_033487</td>
<td>CDC2L1</td>
<td>Cell division cycle 2-like 1 (PITSLRE proteins)</td>
<td>69.7</td>
<td>65.6</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_003718</td>
<td>CDC2L5</td>
<td>Cell division cycle 2-like 5 (cholinesterase-related cell division controller)</td>
<td>34.7</td>
<td>39</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_001260</td>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
<td>47.3</td>
<td>42.1</td>
<td>↑ or ↓</td>
<td>15, 17, 19, 22</td>
</tr>
<tr>
<td>NM_001286</td>
<td>CDK8</td>
<td>Cyclin-dependent kinase 8</td>
<td>36.7</td>
<td>35.3</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_001896</td>
<td>CSNK2A2</td>
<td>Casein kinase 2, alpha prime polypeptide</td>
<td>45.3</td>
<td>37.3</td>
<td>↑</td>
<td>16</td>
</tr>
<tr>
<td>NM_022740</td>
<td>HIPK2</td>
<td>Homeodomain interacting protein kinase 2</td>
<td>33</td>
<td>49.4</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>XM_498294</td>
<td>LOC392265</td>
<td>Similar to Cell division protein kinase 5 (T protein kinase II catalytic subunit)</td>
<td>61</td>
<td>52</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell death</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_004119</td>
<td>FLT3</td>
<td>Fms-related tyrosine kinase 3</td>
<td>50.7</td>
<td>44.7</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_002625</td>
<td>PKFB1</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1</td>
<td>34.4</td>
<td>44.4</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_006212</td>
<td>PKFB2</td>
<td>6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2</td>
<td>52.1</td>
<td>38.3</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_005992</td>
<td>MUSK</td>
<td>Muscle, skeletal, receptor tyrosine kinase</td>
<td>36.1</td>
<td>41.2</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_032409</td>
<td>PINK1</td>
<td>PTEN induced putative kinase 1</td>
<td>44.6</td>
<td>40</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_016457</td>
<td>PRKDC</td>
<td>Protein kinase D2</td>
<td>38.4</td>
<td>52.4*</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_022445</td>
<td>TPK1</td>
<td>Thiamin pyrophosphokinase 1</td>
<td>37.8</td>
<td>54*</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lipid metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_153273</td>
<td>HIPK1</td>
<td>Inositol hexaphosphate kinase 1</td>
<td>31.2</td>
<td>45.5</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_018323</td>
<td>P4K2B</td>
<td>Phosphatidylinositol 4-kinase II β</td>
<td>40.3</td>
<td>34.2</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_004570</td>
<td>PIK3C2G</td>
<td>Phosphoinositide-3-kinase, class 2, γ polypeptide</td>
<td>44.4</td>
<td>44.9</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_005027</td>
<td>PIK3R2</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 2 (p85 β)</td>
<td>54</td>
<td>50.5</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>MAPK signaling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NM_002756</td>
<td>MAP2K3</td>
<td>Mitogen-activated protein kinase 3</td>
<td>32.4</td>
<td>43*</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_005922</td>
<td>MAP3K4</td>
<td>Mitogen-activated protein kinase 4</td>
<td>34.5</td>
<td>45.3</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_005456</td>
<td>MAP81P1</td>
<td>Mitogen-activated protein kinase 8 interacting protein 1</td>
<td>42.3</td>
<td>49.4</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_015133</td>
<td>MAP81P3</td>
<td>Mitogen-activated protein kinase 8 interacting protein 3</td>
<td>43.4</td>
<td>54.1</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>NM_024117</td>
<td>MAPKAP1</td>
<td>Mitogen-activated protein kinase associated protein 1</td>
<td>34.4</td>
<td>48.2</td>
<td>↓</td>
<td>19</td>
</tr>
</tbody>
</table>

(Continued on the following page)
PLK1 as New Therapeutic Target for Rhabdomyosarcoma

Table 2. Active kinases identified in the siRNA library screen and their status in primary tumors (Cont’d)

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Symbol</th>
<th>Gene description</th>
<th>Growth inhibition, % (sequence C)</th>
<th>Growth inhibition, % (sequence D)</th>
<th>Gene expression status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_178510</td>
<td>ANKK1</td>
<td>Ankyrin repeat and kinase domain containing 1</td>
<td>53</td>
<td>40.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_005876</td>
<td>APEG1</td>
<td>Aortic preferentially expressed protein 1</td>
<td>52.4</td>
<td>38.9</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_006383</td>
<td>CIB2</td>
<td>Calcium and integrin binding family member 2</td>
<td>31.9</td>
<td>40.6</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>NM_017729</td>
<td>EPS8L1</td>
<td>EPS8-like 1</td>
<td>47.6*</td>
<td>37.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_145059</td>
<td>FUK</td>
<td>Fucokinase</td>
<td>60.2*</td>
<td>42.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_004712</td>
<td>HGS</td>
<td>Hepatocyte growth-factor-regulated tyrosine kinase substrate</td>
<td>33.8</td>
<td>38</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_020836</td>
<td>KIAA1446</td>
<td>Brain-enriched guanylate kinase-associated protein</td>
<td>36.7</td>
<td>43</td>
<td>↓</td>
<td>19</td>
</tr>
<tr>
<td>XM_292160</td>
<td>MGC7549</td>
<td>Similar to Serine/threonine-protein kinase</td>
<td>49.1</td>
<td>49.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_012395</td>
<td>PFTK1</td>
<td>PFAIRE protein kinase 1</td>
<td>53.8*</td>
<td>36</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_181805</td>
<td>PKIG</td>
<td>Protein kinase (cAMP-dependent, catalytic) inhibitor γ</td>
<td>33.4</td>
<td>45.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_052902</td>
<td>STK11IP</td>
<td>Serine/threonine kinase 11 interacting protein</td>
<td>47</td>
<td>46.2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_015000</td>
<td>STK38L</td>
<td>Serine/threonine kinase 38 like</td>
<td>53.7*</td>
<td>33.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_031432</td>
<td>UCK1</td>
<td>Uridine-cytidine kinase 1</td>
<td>49.5</td>
<td>54.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NM_144624</td>
<td>UHMK1</td>
<td>U2AF homology motif (UHM) kinase 1</td>
<td>46.6</td>
<td>40.7</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE: Gene expression status: -, unknown; ↑ or ↓: overexpression or downregulation in relation to normal muscle as stated in corresponding references.

*Apoptosis is at least 5% more than the control based on nuclear properties (nuclear condensation and higher Hoechst intensity).

Results

siRNA Library Screen Identifies Phosphatases and Kinases Central to the Growth Control of Rhabdomyosarcoma

In this study, we profiled the impact of each human kinase and phosphatase by transfecting RH30 cells with 5 nmol/L siRNA in duplicate using two unique oligonucleotides. Cell growth was assessed 72 hours later by Hoechst 33342 staining using a HCS system. From this screen, we determined that 16 of the 206 phosphatases (∼8% of all the known phosphatases) evaluated played a significant role in proliferation and survival of RH30 cells in vitro (Table 1). The degree of growth inhibition ranged from 30% to 80% and in some instances apoptosis was also observed. Much of the antitumor activity was observed in protein (tyrosine and serine/threonine) phosphatases (Table 1). We queried public databases for evidence of these phosphatases in rhabdomyosarcoma. Notably almost all of these “survival phosphatases” have previously been shown to be either upregulated or downregulated in rhabdomyosarcoma tumor samples compared with normal muscle (Table 1), and they are thus potential therapeutic targets for rhabdomyosarcoma. With respect to the kinase screen, 50 of the 691 kinases (∼7% of the kinase) were shown to play a significant role in the growth and survival of RH30 cells in vitro (Table 2). Knocking down these kinases by siRNAs caused an overall 30% to
80% growth inhibition and/or apoptosis. Most of the active kinases are functionally related to cell cycle, cell death, mitogen-activated protein kinase (MAPK) signaling, and lipid metabolism (Table 2). Approximately one third of the identified kinases are known to be expressed, either downregulated or upregulated, in rhabdomyosarcoma tumor samples (Table 2), yet most of them have not previously been associated with rhabdomyosarcoma. Of particular

Figure 1. Active phosphatases and kinases grouped into functional categories as well as cell signaling pathways by IPA analysis. A, functional categories of phosphatases. B, simplified signaling pathways for phosphatases. C, functional categories of kinases. D, simplified signaling pathways for kinases. Note the small P values and thus large -log(P value) indicating significant association rather than random observation for categories such as cell cycle, cell death, etc. The P value is calculated with the right-tailed Fisher’s exact test according to IPA. Only those that have more Functions/Pathways/Lists Eligible molecules than expected by chance are significant.
PLK1 was one of the most important “survival kinases” for rhabdomyosarcoma identified in this screen. Further validation of this exciting lead is described below.

To understand how the identified phosphatases and kinases are functionally related to one another the data were analyzed using IPA, which employs proprietary databases to establish cell signaling networks based on peer-reviewed publications. The analysis confirmed the functional categories of phosphatases and kinases based on gene ontology, and showed that there is a significant association of the identified genes with functions in cell cycle, cell death, and metabolism (Tables 1 and 2; Fig. 1). Of the 50 kinases identified, many of them are directly or indirectly involved in the MAPK, phosphoinositide 3-kinase (PI3K), and Jnk pathways, which lead to the eventual activation of PLK1 (Fig. 1D). On a genome-wide scale, the present study provides new clues about the growth control of rhabdomyosarcoma cells and important signaling networks involved. This information may lead to the development of novel therapeutic approaches in the future.

Ablation of Identified Phosphatases and Kinases Inhibits the Growth of Both aRMS and eRMS in vitro

Given the molecular heterogeneity of rhabdomyosarcoma and the initial aRMS-based screen, the effects of silencing selected phosphatases and kinases that were identified in the primary screen were further tested on two additional rhabdomyosarcoma cell lines, CW9019 (aRMS) and RD (eRMS). The selected siRNAs targeting 12 phosphatases were re-screened and showed significant growth inhibition on both RH30 and CW9019 cells (Fig. 2A). Similarly, the siRNA silencing of 16 selected kinases inhibited the growth of all three rhabdomyosarcoma cell lines, RH30, CW9019, and RD (Fig. 2B). The results indicate a broad activity of these target genes on rhabdomyosarcoma, and further validate the screening results. It is noteworthy that inhibiting PLK1 consistently proved to have the greatest impact on growth suppression.

Silencing PLK1 Induces Significant Growth Inhibition and Apoptosis of Pediatric Cancer Cell Lines

To validate PLK1 as a potential target, its expression was evaluated in the rhabdomyosarcoma cell lines RH30, CW9019, and RD, and a human pediatric glioblastoma multiforme cell line, SF188, by immunofluorescence and Western blotting. PLK1 was readily detectable in each of these cell lines and was exclusively found in the nucleus (Supplementary Fig. S1). Silencing PLK1 by siRNA caused >80% growth reduction in these four cell lines but not in a mouse myoblast cell line, C2C12 (Fig. 3A). Evidence for the
induction of apoptosis following the loss of PLK1 was independently confirmed using cell-based immunofluorescence assays for p-H2AX S139 and propidium iodide uptake (Fig. 3B). The growth reduction correlates with the loss of PLK1 protein expression by immunoblotting and an induction of apoptosis as indicated by p-H2AX S139 (Fig. 3C). These findings were extended to models representing pediatric Ewing’s sarcoma (TC32), glioblastoma (SF188), and neuroblastoma (IMR32 and KCNR), where silencing PLK1 markedly induced apoptosis based on the induction of p-H2AX S139 (Fig. 3C). Furthermore, we confirmed that silencing PLK1 leads to the induction of apoptosis given the observed activation of caspase 3 and poly(ADP-ribose) polymerase cleavage (Supplementary Fig. S2). In contrast, silencing PLK1 in nontumor mouse myoblast C2C12 cells did not induce apoptosis (Fig. 3C and Supplementary Fig. S2). To explain how the loss of PLK1 leads to growth inhibition and the induction of apoptosis, we report that silencing PLK1 caused decreased levels of p-CDC25C and cyclin B1. There was also an increase in the cell cycle arresting protein WEE1 in contrast to the control (WT).

**PLK1 Is Overexpressed in 10 of 10 Rhabdomyosarcoma Cell Lines and in ~50% of Primary Rhabdomyosarcoma Tumors**

Gene expression microarray analysis revealed that PLK1 was highly expressed in all 10 rhabdomyosarcoma cell lines examined compared with normal (5 years old) or fetal skeletal muscle cells (Fig. 4A). Moreover, PLK1 was present in all primary tumor samples, and it was overexpressed (>1.5 fold increase compared with normal tissue) in 47% and 51% of primary aRMS (17 of 36 samples) and eRMS (21 of 41 samples) tumors (Fig. 4B and C; Supplementary Table S1) based on gene expression analysis. The overexpression of PLK1 applies to subtypes of aRMS regardless of whether or not they express PAX-FKHR fusion proteins. Specifically, PLK1 was overexpressed in 67% (4 of 6 cases) of tumors where neither
Figure 4. Expression of PLK1 mRNA in normal skeletal muscle samples, rhabdomyosarcoma cell lines, primary tumor biopsies of pediatric rhabdomyosarcoma, Ewing’s sarcoma, osteosarcoma, and neuroblastoma as revealed using cDNA microarrays. A, PLK1 overexpression in 10 rhabdomyosarcoma cell lines. B, PLK1 expression in primary aRMS tumor biopsies. C, PLK1 expression in primary eRMS tumor biopsies. D, PLK1 expression in primary tumor biopsies of Ewing’s sarcoma (ESFT), neuroblastoma (NBL), and osteosarcoma (OS) in comparison with rhabdomyosarcoma (ERMS and ARMS) and normal tissues.
PLK1 is commonly expressed in primary rhabdomyosarcoma and is an excellent candidate for targeted therapy.

**PLK1 Is Overexpressed in Primary Tumors of Pediatric Ewing’s Sarcoma, Neuroblastoma, and Osteosarcoma**

When PLK1 expression in primary pediatric tumors of rhabdomyosarcoma, Ewing’s sarcoma, neuroblastoma, and osteosarcoma were compared together in the same study, it was found that all four groups had significantly ($P < 0.001$) higher levels of PLK1 than normal tissues from breast, cerebellum, heart, kidney, liver, muscle, pancreas, prostate, spleen, and thyroid (Fig. 4D). The results support our earlier findings in rhabdomyosarcoma and expanded the potential application of PLK1 in additional pediatric cancers.

**Discussion**

Recently several studies have described the in vivo gene expression profiles of rhabdomyosarcoma, with the aim of associating specific genes that distinguish subtypes of rhabdomyosarcoma either for tumor diagnosis or for tumorigenesis (4, 6, 12–18, 30). Our study represents the first attempt to identify novel therapeutic targets by directly measuring the inhibitory effect of siRNA libraries on the growth of rhabdomyosarcoma cells. As a result, we have identified 16 phosphatases and 50 kinases that play significant roles in the growth control of rhabdomyosarcoma cells. Some of these genes are implicated in rhabdomyosarcoma cells for the first time, whereas others have previously been linked to this disease, including CDK4, PDGFRα, PRKCD, PRKCG, SKP2, etc. (Table 2; refs. 4, 22, 25, 31). Overall these examples illustrate the power of using an unbiased genome-wide screening strategy to identify novel targets, particularly when the results confirm more traditional candidate gene approaches. The screening results further advance our understanding of the growth control of rhabdomyosarcoma cells. In addition, some of these active genes are known to be present in primary tumor samples and are considered important in the tumorigenesis of rhabdomyosarcoma and perhaps growth control. Together this indicates a promising avenue for the advancement of targeted therapies for this rhabdomyosarcoma.

Several cell signaling pathways have been suggested for rhabdomyosarcoma tumors for their involvement in apoptosis, tumor progression, and growth (19). In particular, the PDK-1/AKT, IGF-2/AKT or ERK, PI3K/AKT, mTOR/Hif-1α/VEGF and STAT3 pathways have been implicated in rhabdomyosarcoma (19, 24, 25, 31–35). Consistent with this, we show by IPA analysis that a majority of the active kinases are associated directly or indirectly with MAP/PI3K/hsk pathways, and that these pathways lead to the downstream activation of PLK1 that is one of the most important survival kinases for rhabdomyosarcoma identified in the screen. Such information is crucial in understanding rhabdomyosarcoma in a large context and in designing therapeutic strategies accordingly, as the combined therapy of several key targets may lead to better outcomes of the treatment.

Drug discovery efforts have already been initiated against cell cycle–related kinases, such as cyclin-dependent kinases (CDK), and aurora and polo-like kinase families. These cell cycle protein kinases play critical roles in mitotic entry and chromosome segregation and are often overexpressed in a variety of cancers (36). Inhibition of these proteins frequently results in mitotic arrest and subsequently apoptosis. Therefore, it is not surprising that a large number of the active kinases identified in this study, in particular, PLK1, AUKRB, and CDK4, are in the functional groups related to cell cycle, cell death, or apoptosis. The pharmacologic inhibition of these cell cycle protein kinases may represent a useful therapeutic strategy to control tumor growth and possibly promote myogenic differentiation in rhabdomyosarcoma (22). Clinical trials addressing the efficacy of PLK1 have recently begun for the treatment of adult cancers (37, 38), and therefore similar approaches may be taken to improve the treatment of pediatric rhabdomyosarcoma.

PLK1 is perhaps the best characterized member of the human polo-like family. It acts in both mitotic entry and progression, and plays a key role in cell cycle checkpoint recovery after DNA damage (38). PLK1 is overexpressed in a variety of cancers, such as lung, breast, ovarian, and prostate cancers (36), and is often correlated with poor patient prognosis (38). Numerous studies have now established that PLK1 is a prime target for drug development in proliferative diseases such as cancers (36, 38, 39). However, its significance in childhood cancers has not been reported. PLK1 was identified in our study as one of the most important survival kinases for rhabdomyosarcoma cells in vitro because silencing it resulted in the greatest degree of growth inhibition compared with the other kinases and phosphatases tested. It was also found to be overexpressed in 49% of primary rhabdomyosarcoma tumors ($n = 77$) in this study. Our data further indicate that normal skeletal muscle may not require PLK1 whereas cancer cells do. This is in direct support of the studies that show PLK1 depletion induced apoptosis in cancer cells, whereas normal cells could survive (40, 41). It has been suggested that the loss of PLK1 in primary cells may be compensated by backup kinases and thus is less sensitive to PLK1 depletion (41). In our study, silencing PLK1 in the RH30 cancer cell line led to decreased protein levels of p-CDC25C and cyclin B1, both of which are direct targets of this kinase (42, 43). Conversely, there was an increase in WEE1, a cell cycle inhibitor. These results indicate that the significant growth inhibition of PLK1 silencing on rhabdomyosarcoma cells is likely attributable to cell cycle arrest at G2-M, followed by apoptosis, as is the case in other cancer cell types (41, 44, 45). In addition, there are reports that PLK1 is involved in the inhibition of the mitochondrial-mediated apoptosis pathway by maintaining the stability of antiapoptotic proteins such as survivin, Bcl-2, and Mcl-1 (46). Several studies have shown that induction of apoptosis after PLK1 depletion is independent of the p53 pathway. However, this issue remains somewhat controversial (41, 45, 47). The rhabdomyosarcoma cell lines used in this study express mutant p53 (48, 49), indicating that its status may

Mol Cancer Ther 2009;8(11). November 2009

Published OnlineFirst November 3, 2009; DOI: 10.1158/1535-7163.MCT-09-0365
not be a determinant of sensitivity to PLK1 depletion. Further study is necessary to clarify the apoptotic pathway.

Similar to PLK1, the knockdown of several phosphatases and kinases such as PPP1R12C, SKIP, PRKCD, AURKB, and PTK1 also resulted in significant inhibition of rhabdomyosarcoma cell lines, and these phosphatases and kinases are candidates for further evaluation. Also, for some siRNAs in the libraries, the two siRNA duplexes targeting the same gene showed significant differences in their growth inhibition activity (data not shown). They were not considered further in this study, but deserve future investigation. In addition, it is noted that some of the active genes identified in this study were reported to be downregulated in a few gene profiling studies (16, 17, 19, 22), although silencing them by siRNA caused significant in vitro growth inhibition. The essential roles of these genes in cell culture models of cancer may differ from their actions in primary tumors. There are also conflicting reports of the gene expression status (15–17, 19, 22, 31). Future exploration of these potential therapeutic candidates should be accompanied by confirmation of their in vivo status.

In conclusion, we used a genome-wide rather than candidate approach to search for novel molecular targets for rhabdomyosarcoma. By screening the siRNA libraries of human phosphatases and kinases, we identified 16 phosphatases and 50 kinases that play significant roles in the growth control and survival of rhabdomyosarcoma cells. In particular, PLK1, is one of the most important survival kinases for rhabdomyosarcoma. Silencing it by siRNA caused significant growth inhibition and apoptosis in vitro. More importantly, it is overexpressed in about half of the rhabdomyosarcoma primary tumor biopsies examined regardless of their molecular subtype, and thus holds great promise to be a therapeutic target for rhabdomyosarcoma. The same is true for other types of pediatric Ewing’s sarcoma, osteosarcoma, neuroblastoma, and brain tumors such as glioblastoma multiforme. A recent study reported that PLK1 was an excellent molecular target for the treatment of glioblastoma multiforme. A recent study reported that PLK1 was an excellent molecular target for the treatment of glioblastoma multiforme. A recent study reported that PLK1 was an excellent molecular target for the treatment of glioblastoma multiforme. A recent study reported that PLK1 was an excellent molecular target for the treatment of glioblastoma multiforme. A recent study reported that PLK1 was an excellent molecular target for the treatment of glioblastoma multiforme. A recent study reported that PLK1 was an excellent molecular target for the treatment of glioblastoma multiforme.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Jennifer Law, Jing Wang, and Betty Schaub for technical assistance, as well as the Children’s Hospital Los Angeles Tumor Bank and Dept. of Pathology staff for the provision of tumor samples.

References


Mol Cancer Ther 2009;8(11). November 2009
Molecular Cancer Therapeutics

Small interfering RNA library screen of human kinases and phosphatases identifies polo-like kinase 1 as a promising new target for the treatment of pediatric rhabdomyosarcomas

Kaiji Hu, Cathy Lee, Dexin Qiu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-09-0365

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2009/12/15/1535-7163.MCT-09-0365.DC1

Cited articles
This article cites 49 articles, 18 of which you can access for free at:
http://mct.aacrjournals.org/content/8/11/3024.full#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/8/11/3024.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.