The Homeoprotein Six1 Transcriptionally Activates Multiple Protumorigenic Genes but Requires Ezrin to Promote Metastasis

Yanlin Yu, Elai Davicioni, Timothy J. Triche, and Glenn Merlino

Abstract

The vast majority of deaths associated with cancer are a consequence of a complex phenotypic behavior, metastasis, by which tumor cells spread from their primary site of origin to regional and distant sites. This process requires the tumor cell to make numerous adjustments, both subtle and dramatic, to successfully reach, survive, and flourish at favorable secondary sites. It has been suggested that molecular mechanisms accounting for metastatic behavior can recapitulate those employed during embryogenesis. We have shown that the homeodomain transcription factor Six1, known to be required for normal development of migratory myogenic progenitor cells, is sufficient to promote metastatic spread in a mouse model of the pediatric skeletal muscle cancer rhabdomyosarcoma. Here, we report that Six1 is able to activate the expression of a set of protumorigenic genes (encoding cyclin D1, c-Myc, and Ezrin) that can control cell proliferation, survival, and motility. Although the role of Ezrin in cytoskeletal organization and adhesion has been well studied, the means by which its expression is regulated are poorly understood. We now show that the gene encoding Ezrin is a direct transcriptional target of Six1. Moreover, Ezrin is indispensable for Six1-induced metastasis and highly expressed in a panel of representative pediatric cancers. Our data indicate that Ezrin represents a promising therapeutic target for patients with advanced-stage rhabdomyosarcoma and perhaps other malignancies. (Cancer Res 2006; 66(4): 1982-9)

Introduction

The ability to metastasize is the most devastating of tumor cell behaviors. To successfully metastasize, an opportunistic tumor cell will overcome and survive a series of challenges, including detachment from the primary tumor mass, invasion into blood or lymphatic vessels, transportation to a new site, migration through the endothelium, penetration into surrounding tissue structures, and colonization through sustained growth (1–3). Throughout this process, tumor cells must modify the manner in which they interact with both other cells and to the extracellular matrix (ECM). It has been noted that the behavior of metastatic tumor cells is reminiscent of migratory embryonic cell types, raising the possibility that the two behaviors may share common regulatory mechanisms. In fact, many genes with known essential developmental roles are mutated or aberrantly expressed in advanced tumors.

The process by which tissues and organs form is orchestrated through the regulation of complex behaviors in which cells change their relative positions in the embryo, ultimately becoming associated with other cell types. Skeletal muscle development, for example, requires somitic mesenchymal progenitor cells to successfully migrate from the hypaxial dermomyotome to distant sites where they survive to establish major muscle tissue in the body and limbs. This choreographed series of steps, like metastasis, requires precise modifications of cell-cell and cell-ECM interactions. Delamination, migration, and survival of the muscle progenitor cells are regulated by several factors, including Pax3, c-Met, Lbx1, Mox2, and Six1 (4, 5).

Six1 is a vertebrate homologue of the sine oculis gene, first identified in Drosophila, where it functions in concert with eyeless (Pax), eyes absent (Eya), and dachshund (Dac) to regulate eye development (6). Sine oculis is expressed largely in migrating cells associated with invagination, and its absence is incompatible with Drosophila life (7). Six1 is a member of the Six1 family of homeodomain proteins known to transcriptionally activate muscle genes during myogenesis. Mice deficient in Six1 exhibit abnormal development of skeletal muscle and other organs and do not survive (8–12). Six1 has also been implicated in the autosomal developmental disorder branchio-oto-renal syndrome (13), the regulation of cellular proliferation at the G2-M cell cycle checkpoint (14, 15), and is overexpressed and can be amplified in some cancers (14, 16–18). Recently, we reported the use of microarray expression profiling to identify Six1 as a regulator of metastasis in a mouse model of the pediatric cancer rhabdomyosarcoma, although the mechanism by which Six1 stimulated metastasis was unknown (19). Another prometastasis protein, Ezrin, was identified in the same microarray screen whose expression seemed to correlate with that of Six1.

Ezrin, encoded by Vil2, is a member of the Ezrin-Radixin-Moesin (ERM) family within the band 4.1 superfamily. Ezrin serves as a physical link between the plasma membrane and the actin-based cytoskeleton; however, it has also been implicated in signal transduction pathways involving protein kinase A, Rho, phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase (MAPK), and Src (20–23). Ezrin activity is dictated by its phosphorylation status, and Ezrin is a target of the tyrosine kinase receptor c-Met (24, 25). Ezrin is known to influence cell-cell and cell-ECM interactions through its association with CD44, intercellular adhesion molecules, E-cadherin, and β-catenin (26). Ezrin thus regulates normal cellular morphology, motility, invasiveness, and adhesive ness, all functions that could be subverted by the metastasizing cancer cell. The placement of Ezrin at the nexus of these critical pathways would suggest a significant role in cancer and its progression. In fact, Ezrin had been implicated in the metastasis...
of mammary and pancreatic adenocarcinoma (27, 28) and osteosarcoma (29, 30) as well as in our rhabdomyosarcoma model (19). Although much is known about how Ezrin functions, very little is known about how expression of Vl2 is regulated.

Here, we show for the first time that the developmental homeoprotein Six1 transcriptionally activates Ezrin as well as the cell cycle regulator cyclin D1. Notably, RNA interference (RNAi)–based knockdown of Ezrin fully inhibited the ability of Six1 to promote metastasis in rhabdomyosarcoma cells. The discovery of a key role for Ezrin and perhaps other ERM members in this metastatic pathway, as well as in the response of cancer cells to chemotherapy (31, 32), identifies a family of candidate therapeutic targets for advanced-stage disease.

Materials and Methods

Cell culture, transfection, and antibodies. All rhabdomyosarcoma cell lines were derived from rhabdomyosarcoma tumors arising in hepatocyte growth factor/scatter factor (HGF/SF)-transgenic, Ink4a/Arf-deficient

Figure 1. Six1 binds to the Ezrin gene (vl2) promoter and regulates Ezrin expression. A, Ezrin protein expression was analyzed by Western blotting in the indicated stable rhabdomyosarcoma cell lines transfected with either a Six1 or a shRNA (shSix1) expression vector. Ectopic Six1 expression stimulated Ezrin expression in RMS772, whereas knockdown of endogenous Six1 through a RNAi mechanism inhibited Ezrin expression in RMS14. C, empty vector control. B, the physical interaction of Six1 to chromatin containing the Ezrin promoter was assessed by ChIP assay. Flag-tagged Six1 ectopically expressed in RMS772 cells (a) and native Six1 in RMS14 cells (b) were analyzed using anti-Flag M2 and anti-Six1 antibodies, respectively. Six1 bound to the −1,106 to −870 region of the Ezrin promoter, containing the MEF3-like motif TCAGGG, but not to the irrelevant −230 to −121 region. Inp, input; M, markers. C, Ezrin promoter (−1,616 to −1) activity was shown to be responsive to increasing amounts of a Six1 expression vector (in μg) using a firefly luciferase (Luc) reporter. D, using the same luciferase assay, addition of shSix1 expression vector inhibited luciferase activity driven by the Ezrin promoter. E, a nonfunctional Six1 deletion mutant consisting of only the COOH-terminal end (Flag-Six1-CT) cannot stimulate Ezrin promoter-driven luciferase expression. F, a 237-bp fragment of the Ezrin promoter (−1,106 to −870) shown to bind Six1 by ChIP assay (B) was sufficient to induce full luciferase activation by ectopic Six1 expression; an irrelevant downstream 110-bp fragment (−230 to −121) was not. G, the ability of this 237-bp fragment to stimulate Ezrin promoter activity was greatly reduced when its MEF3-like motif TCAGG was deleted. H, EMSA showing that purified Six1 can bind to an oligonucleotide containing this MEF3-like motif. This binding is competed by a 50-fold excess of cold probe. Arrow, Six1-DNA complex; arrowhead, Six1 antibody supershifted band.
mouse (19) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). Swiss3T3 cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM plus 10% FBS. The mouse Six1 expression plasmid was a generous gift from Dr. Pascal Maire (Institut Cochin, INSERM, Paris, France). Flag-Six1 and Flag-Six1-CT plasmids were constructed by placing the full-length Six1 and the Six1 CT (182-284 amino acids) fragments into pCLFLAG-myc CMV26 (Sigma, St. Louis, MO); the CT fragment encodes the COOH-terminal end, missing both homeodomain and SIX domain. For stable expression of short hairpin RNA (shRNA), dsDNA directed against nucleotides 174 to 194 and 135 to 155 of the mouse ezrin (XM_123004) and six1 (X80339) coding regions, respectively, were synthesized and cloned into the pSUPER vector (19). The luciferase reporter system was constructed using pGL3 luciferase reporter vectors (Promega, Madison, WI). The deletion of the Six1 binding core sequence (TCAGG) in the Ezrin promoter was done using the Quikchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Transfection assays were done using LipofectAMINE 2000 (Invitrogen). Immoblotts were done on lysates generated from cultured cells and tissues solubilized in radioimmunoprecipitation assay buffer (33). Antibodies used included anti-Ezrin (Upstate Biotechnology, Charlottetown, VA); anti-Six1, anti-β-actin, anti–c–cyc1 D1, anti–c–cyc1 A (recognizes both A1 and A2), anti–cyc1 B1, anti–cyc1 E1, and anti–c–cyc1 Myc (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Flag M2 (Sigma); anti-phospho-Akt(Ser473)4E2 monoclonal, anti-Akt polyclonal, anti-phospho-p42/44, and anti-p42/44 (Cell Signaling, Beverly, MA).

Luciferase reporter assays. Luciferase assays were done in 24-well plates in triplicate. Cells (4 × 10^4) were seeded into 24-well plates 1 day before transfection. At 24 hours post-transfection, the cells were harvested and lysed in 200 μL cell lysis buffer (PharMingen, San Diego, CA). The luciferase activity was measured using the Lumat LB 9507 (Wallac, Inc., Gaithersburg, MD) with 70 μL A L cell lysis buffer (PharMingen). The luciferase values were normalized using β-galactosidase activity as an internal control. Ezrin promoter fragments used in luciferase reporter assays included the full –1,616 to –1 region, the –1,106 to –870 region containing the MEF3-like motif TTCAGGA, and the –230 to –121 control region. The –94 to –1 cyclic D1 promoter fragment was used for the luciferase reporter.

Adhesion assay. The adhesion assay was developed based on previously described assays (34, 35). All adhesion assays were done in 96-well plates in triplicate. Fibronectin (100 μL; 10 μg/mL Sigma) was added to the appropriate wells, leaving sufficient blank wells to determine 100% attachment, were gently washed thrice with 100 μL A L water, 0.1% (w/v) crystal violet solution (100 μg/mL) was added to each sample well and incubated for 20 minutes at room temperature. After washing the samples with 200 μL A L of the reaction were run in a 2% SDS-PAGE gel, and the bands were visualized by using a Typhoon Imager (Amersham, Piscataway, NJ). Band intensities were quantified by using the ImageQuant software (Molecular Dynamics). Total RNA was isolated from cells using TRIzol reagent (Gibco). RNA concentration, purity, and integrity were determined by UV spectrophotometry. Total RNA (2 μg) was incubated with 30 ng random primer at 42 °C for 30 minutes in a final volume of 20 μL reaction mixture containing 1 × reaction buffer, 5 μmol/L deoxynucleotide triphosphate (dNTP), 10 mmol/L DTT, 0.5 unit/μL RNase (Promega), and 200 units SuperScript Rnaase H Moloney murine leukemia virus reverse transcriptase (Invitrogen); reaction mixtures were incubated at 95 °C for 10 minutes. Following reaction mixtures (1 μL) were amplified in 20 μL PCR reaction mixture containing 1 × PCR reaction buffer, 1.5 mmol/L MgCl₂, 100 μmol/L dNTP, 5 pmol primers, and 1 μL 18S rRNA primer set (Ambion, Austin, TX) as internal standards and 1 unit Taq DNA polymerase (Invitrogen) for 30 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. Following PCR, 10 μL of the reaction were run in a 2% agarose gel, the PCR bands were imaged using Eagle Eye II (Stratagene), and data were analyzed using NIH image software (the sense and antisense primers used were GGAGAACACCGAAAACAATAAC and GGCGTGCTTGGAAAGGGC for mouse Six1, respectively, and TGCTTTGCTGAAAAGC-TAGGC and CACCTTTGAGATGAAAATCC for cyclic D1, respectively).

Experimental metastasis assays. For tail vein injection assays, 10^6 cells were i.v. injected via the tail vein of 5- to 6-week-old male athymic nude mice. Tumor numbers were obtained by visual inspection of tissues in mice euthanized 21 days post-transplantation (19, 33).

Oligonucleotide microarray analysis. Frozen tumor samples were obtained from the Pediatric Cooperative Human Tissue Network tumor bank (Columbus, OH) and the Children’s Hospital Los Angeles institutional tumor bank. Histopathologic diagnoses for rhabdomyosarcoma tumors were based on the International Classification of Rhabdomyosarcoma criteria (37). All data management and analysis were conducted using the Genetrix suite of tools for microarray analysis (Epicerent Software, Pasadena, CA). Probe set modeling and data preprocessing were derived using the ProbeProfiler algorithm (Corimbia, Berkeley, CA). The Affymetrix probe set modeling and data preprocessing were derived using the ProbeProfiler algorithm (Corimbia, Berkeley, CA). The Affymetrix (Santa Clara, CA) U133A GeneChip tumor microarray data set, sample clinical covariates, and microarray protocols can be found on the National Cancer Institute Director's Challenge3 and University of Southern California/Children’s Hospital Los Angeles Genome Core4 Web sites. ANOVA was also used for statistical analysis of differential gene expression between rhabdomyosarcoma subtypes and other soft-tissue sarcomas.

Electromobility shift assay. Two complementary oligonucleotides containing the Six1 binding core sequences were annealed and the recess 3'-end filled with [α-32P]dTTP in the presence of DNA polymerase (Klenow fragment; ref. 36). Recombinant human Six1 protein (50 ng), purchased from Abnova (Taiwan), were used in per binding assay. Electromobility shift assay (EMSA) was done according to the manufacturer's instructions (Panomics, Redwood City, CA) using 2 ng probe and 1 μg anti-Six1 antibody (A-20, Santa Cruz Biotechnology). Competition was done using 100 ng cold probe. Sequences from the Ezrin and cyclin D1 promoters used for the EMSA were CCCCAATAGAAATTTACGGGACAGTCG and GGGATTCCTTTAAGTTTCAGATCCCCCTTGG, respectively.

Chromatin immunoprecipitation. To prepare chromatin, cells were formaldehyde cross-linked for 15 minutes at room temperature by adding 0.1 volume of cross-linking solution directly to the culture medium in the plates. Cross-linking was stopped by the addition of glycine to a final concentration of 125 μmol/L. Cells were washed twice with ice-cold PBS, harvested in PBS by scraping, and subjected to chromatin immunoprecipitation (ChIP) analysis following the manufacturer's instructions (Upstate Biotechnology). Immunoprecipitated RNAs were analyzed by PCR using the following primers: Ezrin promoter –1,106 to –870, GTAGCAAAAGGCTC-CACAG and ACCCTTCCAGTGCAGTACC; Ezrin promoter –230 to –121, ATCCCAAGTTTGTGAAGAAGAG and GCAGGTTCCTACCTGTGGTGA; cyclin D1 promoter –919 to –614, AGGACAAATCAGATCCCCAC and GACC-CTTTGCTGAAATCC; and cyclin D1 promoter –233 to –103, TTTCCTGCTCCGGGTTCG and GTCTGGATGCTCTGACTGTAC.

Relative quantitative reverse transcription-PCR. Total RNA was extracted from cells using TRIzol reagent (Gibco). RNA concentration, purity, and integrity were determined by UV spectrophotometry. Total RNA (2 μg) was incubated with 30 ng random primer at 42 °C for 30 minutes in a final volume of 20 μL reaction mixture containing 1 × reaction buffer, 5 μmol/L deoxynucleotide triphosphate (dNTP), 10 mmol/L DTT, 0.5 unit/μL RNase (Promega), and 200 units SuperScript Rnase H Moloney murine leukemia virus reverse transcriptase (Invitrogen); reaction mixtures were incubated at 95 °C for 10 minutes. Reaction mixtures (1 μL) were amplified in 25 μL PCR reaction mixture containing 1 × PCR reaction buffer, 1.5 mmol/L MgCl₂, 100 μmol/L dNTP, 5 pmol primers, and 1 μL 18S rRNA primer set (Ambion, Austin, TX) as internal standards and 1 unit Taq DNA polymerase (Invitrogen) for 30 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds. Following PCR, 10 μL of the reaction were run in a 2% agarose gel, the PCR bands were imaged using Eagle Eye II (Stratagene), and data were analyzed using NIH image software. The sense and antisense primers used were GGAGAACACCGAAAACAATAAC and GGCGTGCTTGGAAAGGGC for mouse Six1, respectively, and TGCTTTGCTGAAAAGC-TAGGC and CACCTTTGAGATGAAAATCC for cyclic D1, respectively.

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4. http://genom core.chla.usc.edu/GenomeCore/GenomeCore.html.
Results

Six1 transcriptionally activates the gene encoding Ezrin. We reported previously that Six1 and Ezrin were both up-regulated in highly metastatic mouse rhabdomyosarcoma cell lines relative to those that were poorly metastatic. Furthermore, we found that Ezrin expression seemed to correlate with Six1 expression in human rhabdomyosarcoma and posited that they might be acting in the same pathway (19). To test this notion, the effect of altering Six1 expression levels on Ezrin expression was examined. Figure 1A shows that when a Six1 expression vector was introduced into the poorly metastatic cell line RMS772, which expresses low levels of both Six1 and Ezrin, Ezrin expression was enhanced. Conversely, when a Six1 shRNA expression vector was used to knockdown expression of Six1 expression in the highly metastatic cell line RMS14, which expresses relatively high levels of both Six1 and Ezrin, Ezrin expression was reduced (Fig. 1A).

These data indicate that Six1 can regulate Ezrin expression and raise the possibility that this homeoprotein transcriptionally activates the gene encoding Ezrin (vil2). To determine if Six1 directly binds to the Ezrin promoter in chromatin, a ChIP assay was done. Three candidate Six1 binding sites consisting of MEF3-like motifs (38) were found within the 5'-flanking region (between −1,616 and −1 bp) of the Ezrin promoter. Figure 1B shows that Six1 can bind to the Ezrin promoter between −1,106 and −870, a region containing the MEF3-like motif TTCAGGA, but not to an irrelevant site near the transcriptional start site. Binding was readily detected using either native Six1 or a Flag-tagged form. To determine if Six1 can stimulate Ezrin transcription, an Ezrin promoter-luciferase reporter construct was employed. Figure 1C shows that Six1 can stimulate luciferase activity in RMS772 cells harboring an Ezrin promoter-luciferase expression vector in a dose-dependent fashion, an effect that can be reversed with a Six1 shRNA expression vector (Fig. 1D). Moreover, an inactive Six1 mutant failed to stimulate luciferase activity in these same RMS772 cells (Fig. 1E). The 237-bp fragment of the Ezrin promoter, identified by ChIP assay to bind Six1, was found to be sufficient to induce full luciferase activation by ectopic Six1 expression (Fig. 1F). However, deletion of the TCAGG binding site in this fragment blocked the ability of Six1 to activate the Ezrin promoter (Fig. 1G). Moreover, EMSA showed that Six1 binds to an oligonucleotide containing this Ezrin promoter MEF3-like motif sequence (Fig. 1H).

Six1 can regulate proliferation through induction of cyclin and Myc. We and others have shown that Six1 can stimulate cellular proliferation (14, 15, 19, 39), an observation that prompted an examination of the effects of Six1 on expression of regulators of the cell cycle in several cell types. Figure 2A shows that ectopic Six1 expression in Swiss3T3 fibroblasts up-regulated expression of two cyclins, cyclin D1 and cyclin A, but not cyclin B1 or cyclin E1. Six1 was also able to up-regulate cyclin D1 expression in rhabdomyosarcoma tumor cells, although the background expression level was higher than that observed in fibroblasts (Fig. 2B). We found that there was a significant correlation (P < 0.001) between the expression of Six1 and cyclin D1 in our original panel of rhabdomyosarcoma cell lines derived from the HGF/SF-transgenic, Ink4a/Arf-deficient mouse (refs. 19, 40; Fig. 3A). Two candidate Six1 binding sites were found within the cyclin D1 gene (ccnd1) 5'-flanking region. A ChIP assay was done to show that Six1 binds to the cyclin D1 promoter between −919 and −614, a region containing the two MEF3-like motifs (TCAGAT and TTAGCAG), either as native Six1 or as a Flag-tagged form (Fig. 3B). In contrast, an irrelevant sequence near the transcriptional start site did not bind Six1. Transcriptional activation of cyclin D1 by Six1 was shown using a cyclin D1 promoter-luciferase construct; activity was competed by expression of Six1 shRNA (Fig. 3C). Figure 3D shows that a nonfunctional Six1 mutant cannot induce luciferase activity in this assay. Furthermore, EMSA showed that Six1 binds to an oligonucleotide containing the cyclin D1 promoter MEF3-like motif TTCAGAT (Fig. 3E).
Because Six1 was found to regulate the cell cycle through genes encoding the cyclins, we examined the effect of Six1 on expression of another cell cycle regulator, c-Myc. Figure 4A shows that Six1 also induced c-Myc expression in RMS772 cells as well as other rhabdomyosarcoma cell lines (data not shown).

**Six1 requires Ezrin to promote metastatic dissemination.** Our data show that the homeoprotein transcription factor Six1 is able to activate a set of protumorigenic agents. We reported previously that Ezrin, as well as Six1, was a critical factor in rhabdomyosarcoma cell metastasis (19). To determine to what extent Six1-mediated metastasis was dependent on Ezrin, an Ezrin shRNA expression vector was introduced into RMS772 cells whose metastatic potential had already been enhanced through ectopic Six1 expression. Figure 5A shows that, as expected, Six1 significantly enhanced metastatic spread of RMS772 cells to the lung; however, this metastatic potential was found to be fully dependent on Ezrin function. It is worth noting that although Ezrin shRNA was highly effective at knocking down Ezrin expression, it did not appreciably affect expression of the other Six1 protumorigenic transcriptional targets, cyclin D1 and c-Myc (Fig. 4B).

What Six1-mediated cellular behaviors are dependent on Ezrin, and what downstream pathways govern them? Six1 was found to activate pathways that could potentially regulate both tumor cell growth (MAPK; Fig. 2B) and survival (Akt; Fig. 2B). However, ectopic Ezrin expression did not enhance proliferation of cultured RMS772 cells (data not shown). Furthermore, knocking down Ezrin through shRNA expression in Six1-transfected rhabdomyosarcoma cells did not block extracellular signal-regulated kinase 1/2 activation but inhibited Akt phosphorylation (Fig. 4B), consistent with the notion that Ezrin-mediated prosurvival pathways are important for successful metastatic spread in sarcoma (41).

**Figure 3.** Six1 binds to the cyclin D1 gene (ccnd1) promoter and regulates cyclin D1 expression. A, relative quantitative RT-PCR analyses of cyclin D1 and Six1 RNA transcripts in 35 mouse rhabdomyosarcoma cell line samples (19). B, the physical interaction of Six1 to the cyclin D1 promoter in chromatin was shown using a ChIP assay. Flag-tagged Six1 ectopically expressed in RMS772 cells (a) and native Six1 in RMS14 cells (b) were analyzed using anti-Flag M2 and anti-Six1 antibodies, respectively. Six1 can bind to the −919 to −614 region (containing 2 MEF3-like motifs) of the cyclin D1 promoter but not to the irrelevant −233 to −103 region. C, cyclin D1 promoter (−944 to −1) activity was shown to be responsive to increasing amounts of a Six1 expression vector using a luciferase gene reporter. Inhibition of cyclin D1 promoter-driven luciferase by expression of shSix1 was also observed in these cells. D, the Six1 COOH-terminal deletion mutant (Flag-Six1-CT) cannot stimulate cyclin D1 promoter-driven luciferase reporter expression. E, EMSA showing that purified Six1 can bind to an oligonucleotide containing the MEF3-like motif TTCAGAT. Binding is competed with a 50-fold excess of cold probe. Arrow, Six1-DNA complex; arrowhead, Six1 antibody supershifted band.
However, analysis of Akt activity in our original panel of highly and poorly metastatic rhabdomyosarcoma cells revealed no overt correlation with metastatic potential (19).

We did find that RMS772 cells ectopically expressing either Six1 or Ezrin showed a significant increase in ECM-associated adhesion relative to their parental counterparts, suggestive of a role in metastasis (Fig. 5B). Notably, addition of shEzrin to Six1-RMS772 cells blocked the enhanced adherence gained through ectopic Six1 expression (Fig. 5B). This result indicates that as with metastatic potential the effect of Six1 on adhesion is highly dependent on Ezrin.

Relationship between SIX1 and EZRIN in human pediatric solid tumors. Previously, we quantified SIX1 and EZRIN expression by reverse transcription-PCR (RT-PCR) in human rhabdomyosarcoma tissues and found that both genes exhibited significantly elevated expression in rhabdomyosarcoma, correlating with stage of progression (19). To determine if the discovered relationship between SIX1 and EZRIN was unique to the childhood cancer rhabdomyosarcoma, we screened an oligonucleotide microarray data set of pretreatment diagnostic biopsies of primary pediatric solid tumors. We found that whereas Ezrin was highly expressed in most tumors examined, SIX1 overexpression was characteristic of only rhabdomyosarcoma, expressed in both alveolar and embryonal subtypes (Fig. 5C). These data suggest that although SIX1 plays an important role in regulating expression of EZRIN in tumors thought to be derived from skeletal muscle progenitors in which SIX1 is known to play a key developmental role, EZRIN may be involved in other nonrhabdomyosarcoma malignancies, where its expression is apparently controlled by other factors.

Discussion

The homeoprotein transcription factor Six1 was shown recently to strongly influence the metastatic potential of rhabdomyosarcoma cells (19). Here, we report that SIX1, found to be highly expressed in most advanced human rhabdomyosarcoma tumors, can stimulate the expression of a battery of protumorigenic genes, including those that regulate cytoskeletal organization, adhesion, survival, and cell cycle. Interestingly, the coordinated up-regulation in metastatic cells of such key gene products may well reprise the normal role of Six1 in embryonic development. Six1 is expressed in migrating myogenic progenitor cells and is required for the development of most migratory hypaxial muscles, including forelimb muscle, diaphragm, and tongue (8, 9). Six1, along with Six4, was recently reported to control myogenic cell delamination and migration from the somite (42). One could envision a scenario in which conversion to a metastatic state by opportunistic tumor cells would be facilitated by subverting multiple molecular pathways/cellular behaviors through inappropriate expression of a few master transcription factors, such as SIX1. Gene products that are controlled by SIX1 include cell cycle regulators, such as cyclin and Myc family members, as well as Ezrin.

Ezrin, whose expression also correlated with rhabdomyosarcoma progression in our original analysis (19), was found to be a direct transcriptional target of Six1. Significantly, Ezrin was a prime metastasis factor, as the effect of Six1 on other transcriptional targets was insufficient to induce metastasis in an environment deficient in functional Ezrin. The significance of Ezrin in metastasis could be explained through its association with myriad potential prometastasis pathways (20–23). Interestingly, Ezrin is a direct...
Figure 5. Phenotypic effects of Six1 and Ezrin in rhabdomyosarcoma cells and assessment of their expression in a panel of pediatric solid tumors. A, knockdown of Ezrin reverses the prometastatic effects of Six1. RMS772 cells were stably transfected with empty vector (−), Six1 expression vector alone, or Six1 plus shEzrin expressing vectors. Tumor cells were i.v. injected via the tail vein into athymic nude mice, and numbers of metastatic lesions were obtained by visual inspection of lungs. *, \( P < 0.02 \) (Student’s t test). B, ECM-associated adhesion of RMS772 cells was analyzed following stable transfection of Ezrin, Six1, and/or shEzrin expressing vectors. *, \( P < 0.02 \) (Student’s t test). C, expression of SIX1 and EZRN in a panel of human primary pediatric tumors, including alveolar rhabdomyosarcoma samples containing a PAX-FKHR translocation (RMS+), rhabdomyosarcoma samples lacking a PAX-FKHR translocation (RMS−), synovial sarcomas (STS), Ewing’s sarcomas and primitive neuroectodermal tumor (EW), neuroblastoma (NB), and osteosarcoma (OS). Data were obtained from an oligonucleotide microarray analysis. N, number of tissues analyzed for each tumor type.

target of the c-Met tyrosine kinase (25), a receptor frequently implicated in metastatic behavior (43, 44). Aberrant c-Met signaling drives rhabdomyosarcomagenesis in the HGF/SF-transgenic, Ink-4a/Arf-deficient mouse model and is commonplace in human osteosarcoma (43), also reported to be dependent on Ezrin for high metastatic potency (29, 30). It is worth noting that c-Met signaling is also required for myogenic progenitor cell migration (45) and has been suggested as a Six1 transactivation target (42). However, several factors prompted us to consider in particular the role of cell adhesion–associated pathways in metastatic rhabdomyosarcoma cells.

Adhesion-dependent events, which can profoundly affect invasive and metastatic cellular behavior, are mediated through complex, carefully coordinated interactions between the integrin family of transmembrane adhesion receptors and the Rho family of GTPases (46, 47). For example, integrins serve as primary sensors of the ECM environment, and cell-ECM interactions can initiate signaling pathways to the cytoskeleton through Rho members RhoA, Rac1, and CDC42 (48). Previously, we reported significant differences in expression between several integrins (i.e., αv-integrin and β3-integrin) during a cDNA microarray-based screen of highly metastatic and poorly metastatic rhabdomyosarcoma cells; we also found that RhoA was involved in Ezrin-mediated metastatic potential of rhabdomyosarcoma cells (19). Here, we show that Ezrin expression significantly influences the ability of rhabdomyosarcoma cells to adhere to ECM as has been reported in other cellular contexts (49–51). More importantly, Ezrin function was required for Six1-enhanced adhesion, reminiscent of its mandatory role in Six1-enhanced experimental rhabdomyosarcoma metastasis.

The importance of Ezrin in cancer progression was supported by the fact that every type of human pediatric tumor examined expressed relatively high levels of Ezrin. In contrast, Six1 overexpression was generally restricted to skeletal muscle–derived sarcomas. These data suggest that the importance of the role of Six1 in regulating pro-oncogenic factors is greater in tumors derived from a lineage in which it is known to have a critical embryonic role. Other homeodomain transcription factors may operate under similar conditions.

Six1 is known to control transit through the cell cycle; in fact, Six1 expression stimulates proliferation of rhabdomyosarcoma cell lines (19). Six1 has been reported to be critical for G2-M checkpoint control and is also expressed throughout G1-S (14, 15), raising the possibility that Six1 could help regulate both checkpoints of the cell cycle. This notion is supported by data presented here showing that Six1 can up-regulate expression of both cyclin D1 and cyclin A and by the work of Ford et al. that Six1 regulates transcription of cyclin A1 in the mammary gland (15), raising the possibility that Six1 could help regulate both checkpoints of the cell cycle. This notion is supported by data presented here showing that Six1 can up-regulate expression of both cyclin D1 and cyclin A and by the work of Ford et al. that Six1 regulates transcription of cyclin A1 in the mammary gland and in breast tumors (39). This is noteworthy because it indicates that cyclins regulating cyclin-dependent kinases operating at both the G1 checkpoint and at the transition from S to M can be stimulated through the activity of a single transcriptional regulator. However, the regulation of cyclin D1, overexpressed in numerous cancers (52), is complex. Cyclin D1 is up-regulated by growth factor stimulation through the MAPK pathway (53), and this pathway is still required for cyclin D1 expression in rhabdomyosarcoma cells even in the presence of excess Six1 (data not shown). Expression of c-Myc, another critical regulator of cell cycle checkpoint function deregulated in many human tumors, was also enhanced in rhabdomyosarcoma cells through Six1 expression. This result is in agreement with Li...
et al. who showed that expression of c-Myc is stimulated in C2C12 mouse myoblasts ectopically expressing a VP16-Six1 fusion protein and that Six1 binds directly to the c-Myc promoter (9).

The data presented here support the notion that transcription factors that function as key regulators of organogenesis can be exploited by tumor cells derived from such organs to promote their metastatic program. Six1 is expressed in migrating somitic mesenchymal progenitor cells and is required for embryonic skeletal muscle development. In rhabdomyosarcoma cells, Six1 can up-regulate several influential proto-oncogenes to promote proliferation, survival, and metastatic spread. Despite this broad sphere of influence, successful metastasis in rhabdomyosarcoma cells is fully dependent on the Six1 transcriptional target Ezrin, which regulates cytoskeletal organization and adhesion. Our data indicate that Ezrin should be considered as a molecular target for treatment of patients with advanced rhabdomyosarcoma.

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