Src Uses Cas to Suppress Fhl1 in Order to Promote Nonanchored Growth and Migration of Tumor Cells

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Abstract
Anchorage independence and motility are hallmarks of tumor cell growth. Tumor cell growth and morphology can be normalized by contact with nontransformed cells. The Src tyrosine kinase phosphorylates specific sites on the focal adhesion adaptor protein Crk-associated substrate (Cas) to promote nonanchored cell growth and migration. We studied the effects of Src and Cas on the expression of >14,000 genes to identify molecular events that underlie these activities. Gene expression in tumor cells that were normalized by neighboring nontransformed cells was used as an additional filter to identify genes that control metastatic cell growth. This process enabled the identification of genes that play roles in anchorage-independent cell growth and migration. One candidate, four and a half LIM domains 1 (Fhl1), acts as a transcriptional regulator that can associate with cell junctions as well as with the nucleus. We show here that Src phosphorylates Cas to block Fhl1 expression. In addition, suppression of Fhl1 is required for Src to promote tumor cell growth. These data show that Fhl1 is a tumor suppressor gene that acts downstream of Src and Cas to specifically block anchorage-independent cell growth and migration. Moreover, Fhl1 was suppressed in tumors from several human tissues. Thus, identification of how Fhl1 controls fundamental aspects of tumor cell growth and metastasis may lead to the development of novel markers that can be used to diagnose human clinical specimens as well as open innovative avenues of investigations aimed at developing reagents that target cancer cells while minimizing damage to normal cells. (Cancer Res 2006; 66(3): 1543-52)

Introduction
Increased anchorage-independent growth and migration distinguish most cancer cells from their nontransformed precursors (1, 2). The Src kinase phosphorylates Crk-associated substrate (Cas) to promote these fundamental hallmarks of tumor cell growth and migration (3–9). Cas is an important component of the focal adhesion complex signaling network (10) that also includes focal adhesion kinase, Grb2, Shc, and paxillin (11, 12). After phosphorylation by Src, Cas can bind to other proteins, including Crk, phosphatidylinositol 3-kinase, Nck, and phospholipase Cy (13–15). Src phosphorylates Cas on specific tyrosine residues to promote anchorage-independent cell growth and migration. Thus, Src transformation of homozygous null Cas knockout (CasKo) cells does not fully promote their anchorage independence or ability to migrate. These transformed growth characteristics can be conferred to CasKo cells by transfection with wild-type Cas (3, 4, 9). Phosphorylation of Cas on Tyr253 is not required for anchorage-independent growth but is critical for Src to promote cell migration. Therefore, Src-transformed CasKo (CasKoSrc) cells transfected with Cas with Tyr253 mutated to phenylalanine (CasF253Src cells) are anchorage independent but migrate significantly less than Src-transformed CasKo cells transfected with wild-type Cas (CasWtSrc cells; ref. 9). As shown in Fig. 1A, CasKoSrc cells undergo anoikis in suspension, CasF253Src cells are anchorage independent, and CasWtSrc cells are anchorage independent and more invasive than CasF253Src cells. We have taken advantage of these useful cell phenotypes to separately investigate mechanisms that control nonanchored cell growth and migration. We have compared the expression of >14,000 genes in anchored and nonanchored Src-transformed CasKoSrc, CasF253Src, and CasWtSrc cells to identify genes responsible for anoikis, anchorage independence, nonanchored growth, and tumor cell migration.

Cells transformed by a variety of agents, including Src, can be normalized by contact with nontransformed cells (16, 17). As shown in Fig. 1A, we have used genes associated with this process as an additional filter to facilitate a more precise identification of genes directly involved in metastatic cell growth. Expression of some of these genes are suppressed by Src transformation, and then are turned back on as the tumor cells are normalized by contact with nontransformed cells (18). Three of these genes, four and a half LIM domains 1 (Fhl1), serum deprivation response protein (Sdpr), and vascular cell adhesion molecule 1 (Vcam1) were also associated with the migration, nonanchored growth, and anoikis induced by the combined actions of Src and Cas.

In this report, we have focused on the role of Fhl1 as a tumor suppressor gene. Fhl1 can act as a transcriptional regulator (19, 20) that is found at cell junctions as well as in the nucleus (21, 22). Here, we show that (a) Src acts with Cas to block Fhl1 expression, (b) Fhl1 acts downstream of Src and Cas to inhibit nonanchored cell growth and migration, and (c) Fhl1 expression is suppressed in several types of human tumors.

Materials and Methods
Cells and tissues. Cell lines used for these studies are illustrated schematically in Fig. 1A and listed in Supplementary Table S5. Fibroblasts obtained from homozygous null CasKo mice were transfected with empty vectors (CasKoHP), v-Src (CasKoSrc), or Src along with wild-type Cas (CasWtSrc) or Cas with Tyr253 mutated to phenylalanine (CasF253Src) as described previously (9). Cells obtained from Cx43 homozygous null knockout mice (Cx43ko) were transfected with Src (Cx43koSrc) as described previously (18).

The pCMV6-XL5 vector containing full-length cDNA encoding Fhl1 (Origene Technologies, Inc., Rockville, MD) was digested with EcoRI and

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

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Figure 1. Use of Cas and Cx43 knockout cells to investigate mechanisms underlying tumor cell growth and migration. A, CasKo cells (top) can be used to distinguish mechanisms leading to anchorage independence from those leading to tumor cell migration. Nontransformed CasKo or CasHP (empty vector control) cells (blue rectangle) form anchorage-dependent contact inhibited monolayers. CasKoSrc (v-Src transfected) cells (purple hexagon) show some morphologic alterations but remain relatively static and anchorage dependent; these cell undergo anoikis in suspension (light purple circle). Src-transformed CasF253Src (transfected with v-Src and Cas with Y253 mutated to F) cells (red circles) are anchorage independent but do not migrate as well as anchorage-independent CasWtSrc (transfected with v-Src and wild-type Cas) cells (red circles). These cells were used to investigate genes distinctly involved in anoikis, anchorage independence, cell migration, nonanchored growth, and heterologous normalization by comparisons indicated by I, II, III, and IV, respectively. Cx43Ko cells (bottom) were used to investigate genes involved in heterologous normalization of Src-transformed cells (red circle) by neighboring nontransformed cells (blue rectangle). Genes arising from this comparison (V) were used as a subsequent filter for genes identified by analysis of CasKo cells. B, probe sets representing genes not expressed, not altered, or associated with anoikis, anchorage independence, migration, and nonanchored growth. Genes in tumor cells affected by heterologous normalization by contact with adjacent nontransformed cells are further indicated. Genes distinctly involved in anoikis, anchorage independence, cell migration, nonanchored growth, and heterologous normalization by comparisons are indicated by I, II, III, and IV, respectively. C, cluster analysis of expression profiles of Sdpr, Vcam1, Fhl1, and Gfra1 in anchored and nonanchored CasKoSrc, CasF253Src, or CasWtSrc cells. Bottom, bar showing relative intensity. Intensities from two independent experiments are given for each cell type. D, Src induces Gfra1 and promotes tumor cell growth. However, Src requires Cas to suppress Fhl1, which would otherwise inhibit specific aspects of tumor cell growth, including anchorage independence and migration.
Table 1. Gene expression changes associated with anoikis, anchorage independence, migration, or nonanchored growth

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Probe set</th>
<th>Fold change</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes affected by anoikis</strong></td>
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<td></td>
</tr>
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<td>Gpr56</td>
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<td>Cdkn1b</td>
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<td>Cyclin-dependent kinase inhibitor 1B (p27)</td>
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<td>Expressed sequence C78541</td>
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<tr>
<td></td>
<td>Ppan</td>
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<td>Transforming growth factor-β2</td>
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<td>Sdpr</td>
<td>–2.12</td>
<td>Serum deprivation response</td>
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*Fold change given as the ratio of signal intensities of genes expressed in nonanchored anchorage-dependent cells (CasKoSrc) over nonanchored CasKoSrc cells. Genes listed as decreased or increased were also induced or suppressed by Src transformation of Cx43Ko cells, respectively. Vcam1 was induced in Src-transformed cells during their normalization by neighboring nontransformed cells.

† Fold change given as the ratio of signal intensities of genes expressed in nonanchored anchorage-dependent cells (CasKoSrc) over nonanchored anchorage-independent cells (CasF253Src). Genes listed as increased or decreased were also induced or suppressed by Src transformation of Cx43Ko cells, respectively.

‡ Fold change given as the ratio of signal intensities of genes expressed in nonanchored anchorage-independent migratory cells (CasWtSrc) over nonanchored anchorage-independent nonmigratory cells (SrcCasF253). Genes listed as increased or decreased were also induced or suppressed by Src transformation of Cx43Ko cells, respectively.

§ Fold change given as the ratio of signal intensities of genes expressed in nonanchored anchorage-independent migratory cells (CasWtSrc) over nonanchored CasWtSrc cells. Genes listed as increased or decreased were also induced or suppressed by Src transformation of Cx43Ko cells, respectively. Sdpr was induced in Src-transformed cells during their normalization by neighboring nontransformed cells.

XbaI to release the Fhl1 coding sequence. This fragment was inserted between EcoRI and XbaI sites in the pEF4/V5-HisA mammalian expression vector (Invitrogen, Carlsbad, CA). This Fhl1 mammalian expression vector or the empty parental pEF4 vector were transfected into Cx43KoSrc cells to create Cx43KoSrcFhl1 or Cx43KoSrcEF4 cells, and CasWtSrc cells to create CasWtSrcFhl1 or CasWtSrcEF4 cells, respectively. Transfectants were selected by growth in zeomycin (1.2 mg/mL) and cultured as described previously (9, 18). Clones were not taken from any cell lines, thus avoiding potential complications of clonal variation. Human clinical specimens obtained from the Cooperative Human Tissue Network3 were frozen in nitrogen liquid and stored at –80°C. 

Cell growth and migration assays. Cells were analyzed for anchored growth, nonanchored growth, and migration as described previously (9, 18, 23, 24). For growth assays, 20,000 cells were plated in 1 mL in each well of tissue culture–treated 12-well cluster plates (Falcon, Bedford, MA) to determine anchored growth or in ultralow attachment 24-well cluster plates (Corning, Acton, MA) to determine nonanchored growth. Cell numbers were counted by Coulter (Fullerton, CA) counter at the indicated time points.

To measure cell migration, 200,000 cells were plated on six-well cluster plates on cell culture inserts with a 3μm pore size as directed by the manufacturer (Costar) and grown for 72 hours (CasWtSrc cells) or 96 hours (Cx43Src cells). Cells were then separately digested from the top of the membrane, bottom of the membrane, and well under the membrane. Migration and colonization were quantitated as the percentage of cells found at the bottom of the membrane or in the well under the membrane over the total cell number.

Cell movement was also observed by video microscopy on a Zeiss (Thornwood, NY) Axiovert 40 inverted microscope. Images were taken every 5 minutes for 24 hours with a Carl Zeiss AxioCam MRm CCD camera and Axiovision 4.3 analysis software.

Analysis of gene expression. Gene expression in anchored Cx43Ko, Cx43KoSrc, CasKoSrc, CasWtSrc, and CasF253Src cells as well as non-anchored CasKoSrc and CasWtSrc cells was examined by microarray analysis as described previously (18, 23). For cells based on the CasKo line, 1,400,000 cells were plated in 10 mL medium on 10-cm tissue culture dishes (Falcon) or 840,000 cells were seeded in Costar ultralow attachment 24-well plates (140,000 cells in 1 mL/well) to examine anchored or nonanchored cells, respectively. For cells based on the Cx43Ko line, 300,000 cells were plated on polyester membranes (Costar). After 24 hours of incubation, cells were quickly harvested by rubber policeman into microcentrifuge tubes, washed once with PBS, and frozen at −80°C (9, 18, 23). All comparisons were done with cells from parallel cultures to control for variability in reagents or experimental conditions. Duplicate independent experiments were done in all cases (n = 2).

RNA extraction and microarray analysis of gene expression was done with gene chips as described (18). Numerical results were normalized to a base value of 100. Cells from the CasKo line were examined with U74Av2, U74Bv2, and U74Cv2 Mouse Expression Array gene chips (Affymetrix, Tokyo, Japan), whereas cells from the Cx43Ko line were examined with U74Av2, U74Bv2, and U74Cy2

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dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA), Cas (Santa Cruz Biotechnology), glial cell line–derived neurotrophic factor receptor α1 (Gfrα1; Abcam, Cambridge, MA), and active Src kinase (Cell Signaling Technology, Danvers, MA). Mouse monoclonal antiserum was used to detect viral Src (Upstate Biotechnology, Charlottesville, VA) and h-actin (Sigma, St. Louis, MO). Primary antiserum was recognized by appropriate secondary antiserum conjugated to horseradish peroxidase and detected using Enhanced Chemiluminescence Western Blotting Analysis Systems (Amersham Biosciences, Piscataway, NJ). After Western blotting, membranes were stained with India ink to verify equal loading and transfer (9, 18).

**Immunofluorescence microscopy.** Cells (n = 300,000) were plated on 35-mm poly-d-lysine–coated glass-bottomed culture dishes (MatTek, Inc., Ashland, MA). For coculture experiments, 300,000 nontransformed cells were plated with 50,000 Src-transformed cells as described previously (18). After 24 hours of growth, cells were fixed with 0.2% formaldehyde, permeabilized with 0.2% Triton X-100 in PBS for 10 minutes, and then washed thrice with 0.1% Tween 20 in PBS followed by 1% bovine serum albumin (BSA) in PBS for 30 minutes. The cells were incubated with Fhl1 antiserum (1:5,000) for 1 hour, washed, and then labeled with goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR). Monoclonal antiserum specific for avian Src (Upstate Biotechnology) or Cas (Upstate Biotechnology) was used at 1:100 and labeled with anti-mouse IgG conjugated to Alexa Fluor 595 (Molecular Probes; 1:5,000) to detect v-Src or Cas, respectively.

Before microscopic analysis, cell nuclei were stained with Hoechst 33342 (Molecular Probes). Cells were visualized on a Zeiss Axiovert 40 inverted microscope with a 40 × 1.0 numerical aperture objective and filter packs suitable for Alexa Fluor 488 (excitation, 470 ± 20; emission, 525 ± 25), Alexa Fluor 595 (excitation, 545 ± 15; emission, 620 ± 30), and Hoechst 33342 (excitation, 360 ± 20; emission, 460 ± 25; Chroma Technology, Inc., Boulder, CO).
Results

We used the requirement of Cas tyrosine phosphorylation to investigate mechanisms that underlie anchorage-independent growth and migration of transformed cells. Genes involved in anoikis, anchorage independence, migration, and nonanchored growth were identified by comparing gene expression profiles from anchored and nonanchored CasKoSrc cells (Fig. 1B and C), anchored CasKoSrc and CasF253Src cells (Fig. 1A and B), anchored CasF253Src and CasWtSrc cells (Fig. 1A and B), and anchored and nonanchored CasWtSrc cells (Fig. 1A and B), respectively. Gene expression was analyzed by Affymetrix nucleotide arrays, and initial comparisons were subsequently filtered for consistency with other appropriate cell types as described in Materials and Methods. Cells were harvested 24 hours after plating to examine genes initially affected by Cas-mediated signaling as opposed to detecting more downstream events related to cell growth.

We analyzed >14,000 genes (22,626 probe sets) in two separate experiments (n = 2). Transcripts encoded by ∼51% (11,464 probe sets) of the genes examined were detected (presence call) in both samples of at least one cell type. Among them, as shown in Fig. 1B, alterations in the expression patterns of genes represented by 72, 44, 39, or 26 probe sets were associated with anoikis, anchorage independence, migration, or nonanchored growth, respectively (see Fig. 1B; Supplementary Tables S1-S4).

To increase their experimental validity, genes were further selected for effects consistent with Src transformation of Cx43Ko cells. As described previously, Cx43Ko cells are nontransformed, are anchorage dependent, and grow into contact inhibited monolayers. Cx43KoSrc cells are anchorage independent and migratory compared with their nontransformed Cx43Ko precursors (18). Expression profiles of genes that passed these filtering criteria are listed in Table 1.

We have shown previously that nontransformed Cx43Ko cells can normalize the growth of neighboring Cx43Src cells. The activities of ∼20 genes have been associated with this heterologous growth control phenomenon (18). This comparison was used as an additional filter to select genes involved in metastatic cell growth (V in Fig. 1A and B). As indicated in Fig. 1B, Src and Cas activity significantly affected the expression of three of these genes in Cx43Ko cells: Vcam1, Sdpr, and Fhl1. Figure 1C shows a cluster analysis of the expression profiles of these genes that are boldfaced in Table 1.

Expression of Vcam1 was decreased by Src transformation of Cx43Ko cells and increased during normalization of Cx43KoSrc cells by adjacent nontransformed cells (18). Expression of this gene was also significantly higher in nonanchored CasKoSrc cells relative to anchored cells (Table 1, genes in boldface). In addition

Figure 4. Fhl1 is expressed by Src-transformed cells transfected with an Fhl1 expression vector. A, equal amounts of protein (10 µg/lane) from anchored and nonanchored Src-transformed cells transfected with a mammalian expression vector encoding Fhl1 or the parental empty vector (pEf4) were analyzed by Western blotting. Fhl1 expression levels in Fhl1 transfectants were equivalent to those seen in nontransformed cells without affecting Src kinase activity. B, Fhl1 expression was visualized by immunofluorescent microscopy of CasWtSrc and Cx43Ko cells. Fhl1 was evident in transformed cells transfected with a mammalian expression vector encoding Fhl1. Bar, 50 µm.
to Vcam1, the p27 cyclin-dependent kinase inhibitor was also induced at the start of apoptosis brought about by anoikis of these anchorage-dependent cells (Table 1).

Like Vcam1, the expression of Fhl1 and Sdpr was decreased as a result of transformation of both cell lines and increased in Src-transformed cells normalized by contact with nontransformed cells. However, as shown in Fig. 1C, Fhl1 expression was lower in anchored CasWtSrc cells than CasF253Src cells, whereas Sdpr expression was lower in nonanchored CasWtSrc cells than anchored cells. Thus, Fhl1 was suppressed in migrating anchorage-independent cells, whereas Sdpr was suppressed when these cells were grown in suspension.

As the name denotes, Fhl1 contains four and a half LIM domains. LIM domains contain cysteine-based zinc finger motifs that bind to other proteins (26). In particular, Fhl1 can associate with proteins involved in transcriptional regulation (19, 20) and formation of cell junctions (21, 22). As shown by Western blotting in Fig. 2A, Fhl1 Suppresses Tumor Cell Growth

Figure 5. Fhl1 inhibits anchorage-independent growth and migration of Src-transformed cells. Growth and migration of Cx43KoSrc and CasWtSrc cells transfected with Fhl1 or the empty parental vector were examined. Anchored (A) and nonanchored (B) growth was analyzed by plating 20,000 cells in each well of 12-well tissue culture plates or 24-well low attachment tissue culture plates, respectively. Cells were counted at the time points indicated. Points, mean (n = 3 for anchored growth, migration, and colonization and n = 4 for nonanchored growth); bars, SE (A and B). C, cell migration was quantitated as the percentage of cells that migrated to the bottom of porous membranes. D, colonization was quantitated as the percentage of cells that migrated through porous membranes and attached to the surface ~1 mm below the membrane. Columns, mean (n = 3 for anchored growth, migration, and colonization and n = 4 for nonanchored growth); bars, SE (C and D). Note that in most cases error bars do not extend outside symbols used for growth curves in (A and B). *, P < 0.05; **, P < 0.005 (t test). Fhl1 did not affect anchored growth but did suppress nonanchored growth and, to a greater extent, migration of Src-transformed cells.

Figure 6. Fhl1 inhibits migration of Src-transformed cells during a 24-hour period. Cell migration was examined by video microscopy of CasWtSrc, CasWtSrcFhl1, and CasWtSrcEf4 cells. Asterisks, representative migration distances in the 0- to 24-hour time frames denoting location of start and end points of marked cells.
Src effectively blocked Fhl1 protein production in anchored and nonanchored cells. However, Fhl1 was only suppressed in transformed cells that contained Cas. For example, Fhl1 was suppressed in Cx43KoSrc cells and Src-transformed CasKo cells transfected with Cas (CasWtSrc and CasF253Src) but not in cells that did not express Cas (CasKoSrc; Fig. 2A). Therefore, it seemed that Src required Cas to block Fhl1 expression. Moreover, wild-type Cas enabled Src to suppress Fhl1 more effectively than CasF253 (compare CasWtSrc with CasF253Src in Fig. 2A). Thus, Fhl1 suppression appeared downstream of Cas and correlated with the ability of Src to promote nonanchored growth and migration of transformed cells (Fig. 1D).

As shown in Fig. 2B, results from Fhl1 mRNA assayed by nucleotide arrays correlated well with Fhl1 protein assayed by Western blotting. Cas significantly reduced Fhl1 expression in both assays. Moreover, phosphorylation of Cas on Tyr253 significantly augmented this affect (P < 0.01, t test).

Gfra1 was chosen as a functionally relevant experimental control to test the validity of our approach designed to distinguish key regulators of anchorage-independent growth and migration of tumor cells from genes involved with the growth of nontransformed cells. Gfra1 activates the Ret tyrosine kinase on ligand binding (27–29) to promote neuronal cell growth and survival (30). In contrast to Sdpr and Fhl1, Gfra1 expression was increased by Src transformation. Data from nucleotide arrays (Fig. 1C) and Western blotting experiments (Fig. 2A) show that Src induced Gfra1 protein production in both anchored and nonanchored cells without the aid of Cas. Gfra1 expression was suppressed in nontransformed cells but comparably high in all Src-transformed cells regardless of Cas expression.

The effects of Src and Cas on Fhl1 expression were confirmed by immunofluorescence microscopy. As shown in Fig. 3A and B, Src effectively inhibited Fhl1 expression, and Cas was required for this effect. Robust Fhl1 expression was seen in nontransformed Cx43Ko cells and CasKo cells transfected with empty parental vectors used for Src and Cas expression (CasKoHP cells) as well as in Src-transformed cells that did not express Cas (CasKoSrc cells). However, Fhl1 expression was suppressed in Src-transformed cells that expressed wild-type or mutant Cas (CasWtSrc, CasF253Src, and Cx43KoSrc cells). These studies also revealed that Fhl1 localized to nuclear and cytoplasmic regions of nontransformed cells.

Cas expression was verified in transfected CasKo cells by Western blotting and immunofluorescence microscopy. As shown in Fig. 2A, although Cas seemed to be suppressed in nonanchored Cx43Ko cells, comparable levels of wild-type and mutant Cas were expressed in anchored and nonanchored CasWtSrc and CasF253Src cells, respectively. As shown in Fig. 3C, wild-type and mutant Cas expression was evident in the vast majority of appropriately transfected cells. The cellular expression pattern of Cas seen in CasF253Src and CasWtSrc cells is consistent with that described previously in Cas-transfected CasKo cells (3, 4).

Induction by heterologous growth control was used as a primary criterion behind selection of Fhl1 as a potential tumor suppressor.
gene. This was confirmed at the protein level by immunofluorescence microscopy of Src-transformed cells cocultured with nontransformed cells. As shown in Fig. 3D, nontransformed cells induced Fhl1 protein production in adjacent tumor cells. Robust Fhl1 expression was seen in transformed Cx43KoSrc and CasWtSrc cells cocultured with nontransformed Cx43Ko or CasWt cells, respectively. This contrasts to suppression of Fhl1 in transformed cells grown alone as shown in Fig. 3A and B.

Although Fhl1 was isolated as a migration-associated gene, it was also suppressed in CasF253Src cells that are anchorage independent but migration deficient (Figs. 1-3). Therefore, Fhl1 suppression was expected to be associated with anchorage independence in addition to tumor cell migration. Fhl1 was transfected into transformed cells to investigate its effects on anchorage-independent cell growth and migration. Fhl1 transgene expression was verified by Western blotting. As shown in Fig. 4A, Fhl1 expression in CasWtSrc or Cx43KoSrc cells matched that of nontransformed cells. Immunofluorescence microscopy (Fig. 4B) confirmed this as well as nuclear and cytoplasmic Fhl1 localization in these cells. In addition, as shown in Fig. 4A, Fhl1 expression did not affect Src kinase activity or Cas expression in these transformed cells.

Data presented in Fig. 5A show that Fhl1 did not significantly affect the growth rate or contact inhibition reflected by cell saturation density of anchored Src-transformed cells. However, as shown in Fig. 5B, Fhl1 did decrease the anchorage growth of CasWtSrc and Cx43KoSrc cells. Anchorage-independent growth of Fhl1 transfectants was ~35% less than control transfectants. This effect was significant (P < 0.05, t test) in both cell types.

As predicted, in addition to suppressing anchorage independence, Fhl1 inhibited transformed cell migration. As shown in Fig. 5C, Fhl1 decreased migration of Cx43KoSrc and CasWtSrc cells through porous membranes by ~45% (1.8-fold). As shown in Fig. 5D, Fhl1 also suppressed the movement of transformed cells in a colonization assay that measured cells ability to migrate through a porous membrane, release from their substrata, float through medium, and anchor to colonize on a different surface (see Materials and Methods). Fhl1 decreased the colonization of Cx43KoSrc cells by ~38% (1.6-fold) and CasWtSrc cells by ~43% (1.8-fold). Fhl1 inhibited transformed cell migration and colonization very significantly (P < 0.005, t test).

Fhl1 inhibited nonanchored cell growth and migration in the face of Src activity and Cas expression because these were expressed to equivalent levels in both Fhl1 and control transfectants as shown in Fig. 4A. Suppression of cell migration by Fhl1 was also seen by video microscopy. The motility of representative migrating cells is shown in Fig. 6. Fhl1 transfection inhibited the movement of CasWtSrc cells. Fhl1 transfectants migrated significantly less in a 24-hour period than CasWtSrc cells or control transfectants.

The specific suppression of transformed growth characteristics by Fhl1 led us to investigate its expression in human tumors. Immunohistochemical analysis of human clinical specimens shown in Fig. 7A indicated that Fhl1 expression was suppressed in tumors of the brain, breast, liver, lung, kidney, prostate, and skin. In normal tissue, robust expression was seen in luminal epithelium, which can be the source of tumors found in many tissues.

As presented in Fig. 7B, Fhl1 was detected by Western blotting of normal human brain, breast, and skin. However, Fhl1 was suppressed in metastatic carcinoma samples obtained from human brains that originated from breast and skin. In contrast to Fhl1, Gfra1 was induced in these tumors as well as in normal parietal lobe. Thus, as predicted by our approach, Fhl1 expression was specifically suppressed in metastatic tumors, whereas Gfra1 was increased.

Discussion

Anchorage-independent growth and increased ability to migrate are hallmarks of tumor cell growth. These properties distinguish most cancer cells from nontransformed cells (1, 2). The Src kinase targets integrins and Cas signaling pathways to augment tumor cell growth and motility (31–33). However, molecular events underlying the effects of Src and Cas on cell behavior have not been thoroughly elucidated.

Cas was required for Src to suppress Fhl1 expression but not for Src to augment Gfra1 expression. This serves as evidence for the efficacy of the approach taken here to identify genes specifically involved in anchorage-independent growth and migration that result from the actions of Src in conjunction with Cas. These data are intriguing because they suggest that Gfra1 induction precedes Fhl1 suppression in the events leading to cell transformation. Moreover, the data also suggest that Fhl1 suppression is required for tumor cells to grow in suspension and migrate.

Data here indicate that phosphorylation of Cas inhibited Fhl1 expression as it promoted cell migration. In addition, transfection studies showed that Fhl1 significantly blocked nonanchored tumor cell growth and, to a greater extent, migration. It should be stressed that Fhl1 inhibited specific aspects of tumor cell growth. For example, anchorage independence and motility were suppressed, whereas anchored growth was not affected. Therefore, Fhl1 seems to act as a tumor suppressor rather than as an overall mitotic inhibitor.

The Src kinase remained active during Fhl1 expression. In addition, the Src kinase remained active in the absence of Cas and during normalization of tumor cells by surrounding nontransformed cells (18). These results are consistent with Fhl1 acting downstream of Src. Thus, Fhl1 seems to act as a regulator of tumor cell growth that blocks the link between the effects of Src and Cas on cell growth and motility as outlined in Fig. 1D. Given our Fhl1 expression efficiency in transfected cells of ~80%, it should be noted that these data are likely to represent an underestimate of the effects of Fhl1 on tumor cell growth.

Our results indicate that Fhl1 expression can be suppressed in transformed cells at the mRNA level. The Src kinase can affect a variety of transcriptional control elements. For example, Jaggi et al. found that Src can decrease glucocorticoid receptor-mediated transcription (34). However, Src did not seem to affect glucocorticoid receptor expression in the cells examined here (e.g., Affymetrix probe set 142866_at representing gb:NM_008173). Interestingly, genomic regions from mouse band QA5 and human band Q26.3 of the X chromosome revealed a conserved CpG island that is 845 bases long in mouse and 1,102 bases long in human. This feature begins several hundred bases upstream from the start of transcription and extends a few hundred bases into the transcribed region of the gene encoding Fhl1 in both species (e.g., mouse AK128904 and human BC010998). In addition, other potential modifiers are also suggested from the sequence of this loci. For example, tandem early growth response (EGR) consensus sites (CCGCCCCCGC) are located ~100 bases upstream of the transcriptional start site in both species. Thus, Fhl1 production may be regulated by gene methylation as well as transcription factors, such as EGR.

4 http://genome.cse.ucsc.edu.
Fhl1 expression was found in human skin but suppressed in metastatic melanoma. This is consistent with Fhl1 expression in skin stem cells (35) and its reduction in melanoma cell lines (36). Grossi et al. have reported recently that Fhl1 induction is concomitant with enhanced cadherin adhesion during early keratinocyte differentiation induced by calcium (37). This event is dependent on Rho activity and seems to be mediated by activation of PKN/PRK2 (protein kinase C–related kinase; ref. 37). Our results indicating that Fhl1 acts downstream of Src are consistent with cadherin junctions being interrupted early in the transformation process leading to metastatic cell growth potential. In support of this, Brabek et al. have reported recently that Cas may be phosphorylated to promote invasion and metastasis of Src-transformed cells (32). It should also be noted that the human homologue of Cas, BCAR1, has been implicated in the acquisition of antitoxin resistance by breast cancer cells (38, 39).

Fhl1 is a dynamic transcription factor (19, 20) that can associate with focal adhesions (21) and intercellular junctions (22). Data here indicate that Fhl1 acts downstream of Src and Cas to suppress nonanchored tumor cell growth and migration. Thus, Fhl1 may provide a molecular link between signaling through Src and Cas at cell junctions and the ability of cancer cells to grow in suspension, circulate, migrate, and metastasize.

These investigations employed an experimental design to investigate specific aspects of tumor cell growth. The approach taken here can distinguish mechanisms required for anchorage-independent cell growth and migration from those required for nontransformed cell growth and survival. Therefore, this strategy should be useful for developing reagents that target cancer cells while minimizing damage to normal cells. For example, identification of Fhl1 as a tumor suppressor gene may aid the development of novel reagents that specifically identify and suppress the growth of malignant and metastatic tumor cells.

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References
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