**Src Kinase Is a Novel Therapeutic Target in Lymphangioleiomyomatosis**

Alexey Tyryshkin, Abhisek Bhattacharya, and N. Tony Eissa

**Abstract**

Lymphangioleiomyomatosis (LAM) is a progressive cystic lung disease affecting some women with tuberous sclerosis complex (TSC). Sporadic LAM can develop in women without TSC, owing to somatic mutations in the TSC2 gene. Accumulating evidence supports the view of LAM as a low-grade, destructive, metastasizing neoplasm. The mechanisms underlying the metastatic capability of LAM cells remain poorly understood. The observed behavior of LAM cells with respect to their infiltrative growth pattern, metastatic potential, and altered cell differentiation bears similarity to cells undergoing epithelial–mesenchymal transition. Here, we report increased levels of active Src kinase in LAM lungs and in TSC2−/− cells, caused by a reduction of autophagy. Furthermore, increased Src kinase activation promoted migration, invasion, and inhibition of E-cadherin expression in TSC2−/− cells by upregulating the transcription factor Snail. Notably, Src kinase inhibitors reduced migration and invasion properties of TSC2−/− cells and attenuated lung colonization of intravenously injected TSC2−/− cells in vivo to a greater extent than control TSC2+/+ cells. Our results reveal mechanistic basis for the pathogenicity of LAM cells and they rationalize Src kinase as a novel therapeutic target for treatment of LAM and TSC. Cancer Res; 74(7); 1996–2005. ©2014 AACR.

**Introduction**

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutation in either the TSC1 or TSC2 tumor suppressor genes (1). Lymphangioleiomyomatosis (LAM), a pulmonary manifestation of TSC (2), is a progressive cystic lung disease affecting primarily women of childbearing age. LAM affects 30% to 40% of women with TSC (3, 4) and is characterized by abnormal and potentially metastatic growth of atypical smooth muscle-like LAM cells within lungs and axial lymphatics. Clinical and genetic data suggest a link between the loss of TSC2 function and cell invasion and metastasis. The mTOR is a serine/threonine kinase that positively regulates cell growth, proliferation, and survival (5). Thereby, decreased autophagy due to an activation of the active tyrosine kinase Src, enabling tumor cell invasive growth pattern, metastatic potential, and altered cell differentiation is reminiscent of cells undergoing epithelial–mesenchymal transition (EMT; ref. 14). Src family kinases are nonreceptor tyrosine kinases and key regulators of cellular proliferation, survival, motility, invasiveness, and EMT (15). Signaling through Src kinase suppresses transcription of E-cadherin by upregulating the transcriptional repressors Snail/Slug (16). Collectively, the observed behavior of LAM cells with respect to their infiltrative growth pattern, metastatic potential, and altered cell differentiation is reminiscent of cells undergoing epithelial–mesenchymal transition (EMT; ref. 14). Src family kinases are nonreceptor tyrosine kinases and key regulators of cellular proliferation, survival, motility, invasiveness, and EMT (15). Signaling through Src kinase suppresses transcription of E-cadherin by upregulating the transcriptional repressors Snail/Slug (16). Recent results have shown that, in cancer cells in which the Src pathway is hyperactive, autophagosomes promote degradation of the active tyrosine kinase Src, enabling tumor cell survival (17). Thereby, decreased autophagy due to an activation of mTOR may play a critical role in accumulation of active Src kinase in LAM cells. Hyperactivity of Src has been
implicated in the development of several types of human cancers and in their progression to metastases (18). There are no prior studies addressing potential activation of Src in LAM. Here, we report that Src kinase is activated in LAM cells. In this study, we examined the potential underlying mechanisms of Src activation in LAM cells and tested Src as a novel therapeutic target in LAM.

Materials and Methods

Reagents and antibodies

The following antibodies were used for immunoblot analysis: pSrc(Tyr16), pStat3(Tyr705), Stat3, pErk1/2(Thr202/Tyr204), Erk1/2, S6, pS6(Ser235/236), pFAK(Tyr925), pFAK (Tyr397), mTOR, U0126 (all from Cell Signaling Technology), tuberin, rabbit E-cadherin, MMP9, Snail (all from Santa Cruz Biotechnology), mouse E-cadherin (BD Biosciences), Src (Millipore), pSrc(Tyr418; LifeSpan Biosciences), and HMB45 (Enzo Life Sciences). Src kinase inhibitors PD2 and SU6656 were purchased from Calbiochem. Rapamycin, dasatinib, and saracatinib were purchased from LC Laboratories.

Cell culture and tissue samples

Eker rat embryonic fibroblasts (EEF)4 (TSC2+/−) and EEF8 (TSC2−/−) were maintained in Dulbecco’s Modified Eagle Medium (DMEM)/F12 mixture (1:1) containing 10% heat-inactivated FBS. Lung tissues of normal subjects and subjects with LAM were obtained from the National Disease Research Interchange.

Plasmids, siRNA, and cell transfection

Specific TSC2 (J-003029-11 and J-003029-12), ATG7 (J-0095596-11 and J-0095596-12), and Src (J-080144-11 and J-080144-12) siRNAs were purchased from Dharmacon. Cationic lipid-mediated transient transfection of plasmids was done using Lipofectamine 2000 (Invitrogen).

Immunofluorescence and histochemistry

Cells were grown on glass coverslips, fixed in either cold pure methanol or 4% paraformaldehyde, permeabilized by 0.2% Triton X-100, and blocked in 10% normal goat serum. Primary antibody incubation was done at 4°C overnight in a humidified chamber followed by a 30-minute incubation at room temperature with Alexa Fluor 594–labeled secondary antibodies. Coverslips were mounted by SlowFade gold antifade reagent with 4',6-diamidino-2-phenylindol dihydrochloride (DAPI). Tissue sections were deparaffinized, incubated overnight with primary antibodies at 4°C in a humidified chamber, and then washed and incubated with biotinylated secondary antibodies for 30 minutes at room temperature. Slides were developed using the Vectastatin Elite ABC Kit (Vector Laboratories) and counterstained with hematoxylin. Images were viewed using a Zeiss Axiosvert microscope.

Wound-healing assay

Cells were plated in a 10-cm plate and allowed to form a confluent monolayer that was then scratched with a sterile pipette tip (200 μL), washed with medium to remove floated and detached cells. Wound areas were photographed (magnification, ×100) at the start and 18 hours after treatment to assess the degree of wound closure. Data are expressed in μm² × 1,000.

Cell invasion assay

Cells were studied using Matrigel inserts (BD Biosciences). Serum-deprived cells (5 × 10⁴ cells) were loaded in the upper compartment of the chambers and the bottom wells were filled with chemoattractant (complete media with 10% FBS). After incubation for 18 hours, the membranes were processed and the nonmigrating cells were removed from the upper chamber with a cotton swab and the inserts were fixed with methanol and stained with 1% Toluidine blue. The invading cells were counted in six random fields under a microscope.

Real-time PCR

RNAs were purified using the RNeasy Mini Kit (Qiagen) and cDNA synthesis was performed using the cDNA Reverse Transcription Kit (Applied Biosystems). mRNA expression was measured using a real-time detection system (Applied Biosystems StepOnePlus) in 96-well optical plates using PerfeC-TaqPCR FastMix (Quanta Biosciences). 18S was used as an endogenous control. All analyses were performed in triplicate, and means were used for statistical calculations.

Mouse in vivo imaging

Female CB17-SCID mice, 6 to 8 weeks of age, were purchased from The Jackson Laboratory. Cells were transfected with pGL4.51[luc2/CMV/Neo] vector (Promega), expressing luciferase, using Lipofectamine 2000 (Invitrogen). For intravenous injections, 1 × 10⁶ cells were injected into the retro-orbital vein. Ten minutes before imaging, animals were injected with Luciferin (Promega; 120 mg/kg, i.p.). Bioluminescent signals were recorded using Xenogen in vivo imaging system (IVIS; Xenogen). Total photon flux at the chest regions was analyzed. All animal studies were performed in accordance with protocol approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (Houston, TX).

Statistical analysis

All blots and data are representative of at least three independent experiments. The Student t test was used, and P values of less than 0.05 were considered to be statistically significant.

Results

Enhanced Src activation in LAM lungs

We evaluated tissue samples of lungs of normal subjects and of subjects with LAM. LAM lungs showed collections of LAM cells, which were identified by HMB45 antibodies (Fig. 1A; refs. 11, 19). Phosphorylation of the ribosomal protein S6 was increased in LAM lung tissues compared with normal lungs (Fig. 1B). These data confirm that mTOR is activated in lung tissues of subjects with LAM, as expected to occur secondary to TSC2 deficiency. One of the consequences of mTOR activation is inhibition of autophagy, which was evident by the accumulation of the autophagy substrate p62 (20) in LAM lungs.
Importantly, we found increased phosphorylation of Src on Tyr416 in LAM lung tissues compared with normal lungs (Fig. 1D). These findings were further confirmed by analyzing human LAM lungs by immunofluorescence (Supplementary Fig. S1). Moreover, there was strong correlation between expression of phospho-Src and HMB45-positive cells. However, some HMB45-negative cells contained phospho-Src as well, consistent with the notion that not all LAM cells are HMB45-positive (19). Phosphorylation of Tyr416, in the activation loop of the kinase domain, upregulates Src kinase activity (21). These data suggest that Src is activated in lung tissues of subjects with LAM. To confirm that Src activation in LAM lungs had functional consequences, we tested activation of STAT3, which is a downstream mediator of Src. We found that LAM lungs had elevated phosphorylated STAT3 (Fig. 1E), suggesting that STAT3 is activated in LAM lungs, consistent with a prior report (22).

We then wanted to confirm that the increased activities of Src and STAT3, observed in LAM lungs, were specific to LAM cells. We isolated cells from LAM lung explants and identified LAM cells using antibodies against HMB45. We found that cells positive for HMB45 had increased phospho-(Y416)-Src and phospho-(Y705)-STAT3, whereas non-LAM cells did not exhibit such increase (Supplementary Fig. S2). These data confirm that LAM cells have increased Src and STAT3 activities.

**Src and STAT3 are activated in TSC2−/− cells**

We studied EEFs TSC2+/+ wild-type (EEF4) and TSC2−/− mutant cells (EEF8). These cells are well characterized as a cellular model for LAM and TSC (14, 23). EEF8 cells (TSC2−/−) did not express Tuberin, had increased activity of mTOR, and suppressed autophagy (Supplementary Fig. S3). Activation of mTOR was evident by increased phosphorylation of mTOR and of its substrate p70S6 kinase. Inhibition of autophagy was shown by reduction of LC3-II and by increased level of autophagy substrate p62 protein. Accumulation of p62 in EEF8 cells was not caused by increase of its mRNA. The above data confirmed prior reports that TSC2−/− EEF8 cells have the molecular features of LAM cells. We then investigated whether TSC2−/− cells have increased Src activity. We found that phosphorylation of Src was increased in the TSC2−/− cells (Fig. 2A and Supplementary Fig. S4). We also found that TSC2−/− cells had increased phosphorylated STAT3 (Fig. 2B). Increased STAT3 translocation to the nucleus was observed in TSC2−/− cells (Fig. 2C). Importantly, inhibition of Src by PP2 or Su6656 reduced STAT3 phosphorylation (Fig. 2D). We then wanted to confirm that the increase in Src and STAT3 activities was a direct result of TSC2 deficiency. To this end, siRNA-mediated knockdown of TSC2 in Hela cells resulted in increased Src and STAT3 activities (Fig. 2E), essentially recapitulating the phenotype of TSC2−/− EEF8 cells. That phenotype was also confirmed by the finding of increased phosphorylation of ribosomal protein S6 (Supplementary Fig. S5), which indicated that the activation of mTOR was similar to that observed in EEF8 cells. Moreover, overexpression of Src kinase in wild-type EEF4 cells led to increased Src activity and increased STAT3 phosphorylation (Fig. 2F). These data suggest that TSC2−/− cells have increased Src activity, similar to that found in human LAM lungs. They also show that STAT3 activation in TSC2−/− cells is a downstream event of Src activation.
Enhanced activation of the Src kinase signaling pathway in TSC2−/− cells

The activation of STAT3 in TSC2−/− cells suggested that other Src downstream substrates might also be activated in these cells. One important Src partner is focal adhesion kinase (FAK). We found that levels of FAK were increased in EEF8 cells, likely secondary to increase of its mRNA (Fig. 3A and B). Moreover, we found overphosphorylation of FAK on Y397 and Y925 sites in EEF8 cells (Fig. 3C and D). FAK-Y397 autophosphorylation plays an important role in FAK binding to Src kinase and forming of an active FAK-Src complex (24). Recruitment of Src kinase results in phosphorylation of FAK-Y925 and triggers a Ras-dependant activation of the mitogen-activated protein kinase (MAPK) pathway (25). To evaluate the MAPK pathway activation in EEF8 cells, we examined phosphorylation of Erk. We found increased level of phosphorylated Erk in EEF8 cells, compared with EEF4 cells (Fig. 3E). To determine the effect of Src kinase on Erk phosphorylation, we overexpressed Src kinase in EEF4 cells and we found markedly increased level of Erk phosphorylation (Fig. 3F). To confirm that the increased phosphorylation of Erk in EEF8 was caused by Src kinase, we found that Src-specific siRNA led to a decrease of Erk phosphorylation in EEF8 cells (Fig. 3G). Moreover, Src kinase inhibition by any of four different inhibitors (PP2, SU6656, dasatinib, and saracatinib) reduced Erk phosphorylation in EEF8 cells (Fig. 3H). Taken together, these data indicate the Src signaling pathway is activated in TSC2−/− cells.

Autophagy inhibition results in Src kinase activation

Loss of the TSC2 gene leads to mTOR activation and autophagy inhibition (26). Recently, a role for autophagy has been shown in degradation of active Src (17). We hypothesized that autophagy inhibition in EEF8 contributes to Src activation in these cells. To test this hypothesis, we used siRNA to knockdown autophagy related gene 7 (Atg7) in wild-type EEF4 cells. Atg7 knockdown resulted in inhibition of autophagy as shown by reduction of autophagy marker LC3 type II, and increased active phosphorylated Src (Supplementary Fig. S6A and S6B). Furthermore, mouse embryonic fibroblasts derived from Atg7−/− mice and Atg5−/− mice had increased active Src (Supplementary Fig. S6C and S6D). Finally, treatment of wild-type EEF4 cells with the autophagy-lysosome pathway inhibitor chloroquine resulted in increased active Src (Supplementary Fig. S6E). Thus, autophagy inhibition caused by several independent methods led to accumulation of active Src kinase. Moreover, we found that the phospho-Src levels decreased after induction of autophagy by starvation in TSC2−/− EEF8 cells (Supplementary Fig. S6F). These data suggested that autophagy was involved in modulation of cellular Src kinase activity.

Enhanced activation of the Src kinase signaling pathway in TSC2−/− cells

The activation of STAT3 in TSC2−/− cells suggested that other Src downstream substrates might also be activated in these cells. One important Src partner is focal adhesion kinase (FAK). We found that levels of FAK were increased in EEF8 cells, likely secondary to increase of its mRNA (Fig. 3A and B). Moreover, we found overphosphorylation of FAK on Y397 and Y925 sites in EEF8 cells (Fig. 3C and D). FAK-Y397 autophosphorylation plays an important role in FAK binding to Src kinase and forming of an active FAK-Src complex (24). Recruitment of Src kinase results in phosphorylation of FAK-Y925 and triggers a Ras-dependant activation of the mitogen-activated protein kinase (MAPK) pathway (25). To evaluate the MAPK pathway activation in EEF8 cells, we examined phosphorylation of Erk. We found increased level of phosphorylated Erk in EEF8 cells, compared with EEF4 cells (Fig. 3E). To determine the effect of Src kinase on Erk phosphorylation, we overexpressed Src kinase in EEF4 cells and we found markedly increased level of Erk phosphorylation (Fig. 3F). To confirm that the increased phosphorylation of Erk in EEF8 was caused by Src kinase, we found that Src-specific siRNA led to a decrease of Erk phosphorylation in EEF8 cells (Fig. 3G). Moreover, Src kinase inhibition by any of four different inhibitors (PP2, SU6656, dasatinib, and saracatinib) reduced Erk phosphorylation in EEF8 cells (Fig. 3H). Taken together, these data indicate the Src signaling pathway is activated in TSC2−/− cells.

Figure 2. Activation of Src and STAT3 in TSC2−/− cells (EEF8). A and B, cell lysates of EEF4 and EEF8 were subjected to immunoblot analysis using antibodies against Src kinase and phospho-Y416-Src (A) or STAT3 and phospho-Y705-STAT3 (B). C, EEF4 and EEF8 cells were fixed and immunolabeled by phospho-STAT3 antibodies followed by IgG conjugated to Alexa Fluor 594 (red). Cells were stained with DAPI to visualize nuclei (blue). Graph, percentage of pStat3 (nuclear localization)-positive cells (n = 3). D, EEF4 and EEF8 cells were treated with either DMSO or Src kinase inhibitors PP2 (25 μmol/L), or SU6656 (5 μmol/L) for 4 hours before lysis. Cell lysates were subjected to immunoblot analysis. E, HeLa cells were transfected for 72 hours with control nontarget (NT) siRNA or TSC2-specific siRNA. Cells were lysed and immunoblot analysis was done. Graphs, quantification of phospho-Src and phospho-STAT3, normalized to total Src or STAT3 expression, respectively, n = 3. F, EEF4 cells were transfected for 24 hours with Src kinase or LacZ control plasmids. Cell lysates were analyzed, compared with EEF8, by immunoblotting. Blotting with β-actin antibody was used as a control. Data, mean ± SD. *, P < 0.05; **, P < 0.001. Scale bar, 10 μm.

Enhanced activation of the Src kinase signaling pathway in TSC2−/− cells

The activation of STAT3 in TSC2−/− cells suggested that other Src downstream substrates might also be activated in these cells. One important Src partner is focal adhesion kinase (FAK). We found that levels of FAK were increased in EEF8 cells, likely secondary to increase of its mRNA (Fig. 3A and B). Moreover, we found overphosphorylation of FAK on Y397 and Y925 sites in EEF8 cells (Fig. 3C and D). FAK-Y397 autophosphorylation plays an important role in FAK binding to Src kinase and forming of an active FAK−Src complex (24). Recruitment of Src kinase results in phosphorylation of FAK-Y925 and triggers a Ras-dependant activation of the mitogen-activated protein kinase (MAPK) pathway (25). To evaluate the MAPK pathway activation in EEF8 cells, we examined phosphorylation of Erk. We found increased level of phosphorylated Erk in EEF8 cells, compared with EEF4 cells (Fig. 3E). To determine the effect of Src kinase on Erk phosphorylation, we overexpressed Src kinase in EEF4 cells and we found markedly increased level of Erk phosphorylation (Fig. 3F). To confirm that the increased phosphorylation of Erk in EEF8 was caused by Src kinase, we found that Src-specific siRNA led to a decrease of Erk phosphorylation in EEF8 cells (Fig. 3G). Moreover, Src kinase inhibition by any of four different inhibitors (PP2, SU6656, dasatinib, and saracatinib) reduced Erk phosphorylation in EEF8 cells (Fig. 3H). Taken together, these data indicate the Src signaling pathway is activated in TSC2−/− cells.

Autophagy inhibition results in Src kinase activation

Loss of the TSC2 gene leads to mTOR activation and autophagy inhibition (26). Recently, a role for autophagy has been shown in degradation of active Src (17). We hypothesized that autophagy inhibition in EEF8 contributes to Src activation in these cells. To test this hypothesis, we used siRNA to knockdown autophagy related gene 7 (Atg7) in wild-type EEF4 cells. Atg7 knockdown resulted in inhibition of autophagy as shown by reduction of autophagy marker LC3 type II, and increased active phosphorylated Src (Supplementary Fig. S6A and S6B). Furthermore, mouse embryonic fibroblasts derived from Atg7−/− mice and Atg5−/− mice had increased active Src (Supplementary Fig. S6C and S6D). Finally, treatment of wild-type EEF4 cells with the autophagy-lysosome pathway inhibitor chloroquine resulted in increased active Src (Supplementary Fig. S6E). Thus, autophagy inhibition caused by several independent methods led to accumulation of active Src kinase. Moreover, we found that the phospho-Src levels decreased after induction of autophagy by starvation in TSC2−/− EEF8 cells (Supplementary Fig. S6F). These data suggested that autophagy was involved in modulation of cellular Src kinase activity.
**TSC2 deficiency or overexpression of Src promotes EMT**

To evaluate EMT in TSC2−/− cells, we examined the level and cellular distribution of E-cadherin. We found that the expression and cellular localization of E-cadherin in EEF8 cells were notably altered (Fig. 4A and B). In wild-type cells (EEF4), E-cadherin was readily detectable and localized predominantly at the plasma membrane, in which it is known to play a critical role in adherens junction formation. In contrast, in TSC2−/− cells (EEF8), there was much lower expression of E-cadherin and it did not colocalize with plasma membrane. Instead, most of E-cadherin signals were found in punctate cytosolic structures. One possible explanation for the reduction in E-cadherin in TSC2−/− cells could be due to an increase of its transcriptional repressor Snail. We found marked increase in the expression of Snail mRNA and protein in EEF8 cells (Fig. 4C). Snail activity, measured by its nuclear translocation, was also more pronounced in EEF8 cells (Fig. 4D). Importantly, we observed increased level of Snail in human LAM lungs (Fig. 4E). Furthermore, the increase in Snail expression was limited to LAM cells identified by positive staining for HMB45 (Supplementary Fig. S2C). Matrix metalloproteinase 9 (MMP9), an important marker of EMT, was markedly increased in EEF8 cells (Fig. 4F). To confirm a role for the observed increased Src in TSC2−/− cells in promotion of EMT, we transfected wild-type EEF4 cells with Src and then evaluated several EMT markers. Src overexpression resulted in reduction of E-cadherin and increased levels of Snail and MMP9 (Fig. 4G), essentially recapitulating the phenotype observed in TSC2−/− cells. These dramatic changes in the expression and localization of E-cadherin could account for the decrease in cell adhesion, increased motility, invasiveness, and metastatic potential of TSC2−/− cells.

**Src inhibition reduces EMT markers in TSC2−/− cells**

To validate Src as a potential therapeutic target in LAM, we treated TSC2−/− cells (EEF8) with Src inhibitors dasatinib or saracatinib (27, 28). Both inhibitors reduced levels of Snail, whereas rapamycin had no effect (Fig. 5A and B). Src inhibition also reduced levels of MMP9, as determined by immunoblot analysis and further confirmed by gelatin zymogram (Fig. 5C and D). Overall, dasatinib and saracatinib seemed to have equivalent effects on Src activation (phosphorylation) and on EMT markers. In additional experiments, siRNA-mediated knockdown of Src resulted in decrease of expression of Snail and Mmp9 (Fig. 5E and F). These data are consistent with prior reports of increased immunoreactivity for MMPs in lung biopsy specimens from subjects with LAM and TSC2-deficient LAM-like cells (29–31) and suggest that inhibition or genetic knockdown of Src could reduce EMT in TSC2−/− cells.

**Src inhibition attenuates migration activity of EEF cells**

Using wound-healing assay, we found that inhibition of Src kinase by dasatinib or saracatinib led to reduction of migration...
ability of both EEF8 and EEF4 cells (Supplementary Fig. S7). In contrast, mTOR inhibitor rapamycin had no significant effect on cell migration. It should be noted that the migration assay results reflect, in part, reduction of cell proliferation by Src inhibitors. We found that EEF8 cell proliferation was increased compared with control cells and that Src inhibitors significantly decreased the proliferation of EEF8 cells (Supplementary Fig. S8). These data suggest that Src inhibition is likely to reduce migration ability of TSC2−/− cells.

**Src inhibition reduces invasiveness of TSC2−/− cells**

The invasive properties of EEF4 and EEF8 cells were studied using Matrigel inserts. After incubation for 18 hours, the membranes were processed and the invading cells were counted in six random fields. Invasive cells were counted as the number of migrating cells per field. TSC2−/− cells (EEF8) were much more invasive than control cells (Fig. 6A and B). This behavior is consistent with the notion that TSC2−/− cells have increased invasive and migratory properties, likely secondary to EMT in these cells. The effect of Src inhibition on the invasive properties of EEF cells was evaluated. We found that Src inhibition by dasatinib or saracatinib markedly reduced the invasiveness properties of EEF8 TSC2−/− cells. In contrast, mTOR inhibitor rapamycin had no significant effect. Furthermore, there was no effect of Src inhibitors on the invasiveness in the EEF4 cells, probably because of low invasiveness of these cells (Fig. 6C and D). These data suggest that Src inhibition is likely to reduce invasiveness of TSC2−/− cells and that effect is specific for such cells.

**Src inhibition reduces lung colonization of TSC2−/− cells in vivo**

We evaluated the effect of Src inhibition on the metastatic potential for TSC2−/− cells in vivo. We engineered luciferase-expressing (EEF-Luc) cells to allow in vivo imaging following their injection into mice. EEF8-Luc cells were pretreated with vehicle (dimethyl sulfoxide, DMSO), rapamycin (1 μg/mL), saracatinib (1 μmol/L), or both rapamycin and saracatinib. 1 × 10^6 cells were then intravenously injected into female CB17 SCID mice. Six hours following injection of cells, and 10 minutes before imaging, animals were injected intraperitoneally with 120 mg/kg, luciferin. Bioluminescent signals were recorded using the Xenogen in vivo imaging system (Fig. 7A and B). Total photon flux at the chest region was analyzed. At 24 hours time point after cell injection, mice were sacrificed and their lungs were dissected and imaged in Petri dish (Fig. 7C and D). Saracatinib significantly reduced the number of EEF8-Luc cells that was detected in

---

Figure 4. TSC2 deficiency or overexpression of Src promotes EMT. EEF4 and EEF8 cells or human lungs (E) were analyzed by immunoblots, immunofluorescence microscopy, or RT-PCR (graphs in C and F) to evaluate E-cadherin (A and B), Snail (C–E), or MMP9 (F). B and D, cells were stained with DAPI to visualize nuclei (blue). G, EEF4 cells were transfected for 16 hours with Src or LacZ control vector and analyzed by immunoblot analysis. β-Actin antibody was used as a loading control. Graphs in G indicate the quantification of the immunoblots. Data, mean ± SD; n ≥ 3. *, P < 0.05; **, P < 0.001. Scale bar, 10 μm.
the lungs at 6 and at 24 hours after injection. Rapamycin treatment had no significant effect. Furthermore, we conducted in vivo experiments with injection of luciferase-expressing EEF4 cells treated with DMSO or saracatinib. Saracatinib reduced EEF4 cells lung colonization after 24 hours but not after 6 hours of the cell injection (Fig. 7E–H). Furthermore, although the decrease in lung colonization was significant at 24 hours for both TSC2−/− and TSC2+/+ cells, the extent of reduction was not the same. There was more reduction in TSC2−/− cells (71.3%) than in TSC2+/+ cells (58.7%); compare Fig. 7D and H. Thus, the consequences of Src inhibition were more pronounced in TSC2−/− cells compared with control cells. These results suggest that Src inhibition can reduce the metastatic potential for TSC2−/− cells.

Discussion

This study has three major novel findings. First, Src is activated in LAM cells. Second, Src activation contributes to the pathogenesis of LAM by promoting EMT in TSC2−/− LAM cells. Third, Src inhibition can attenuate the oncogenic and metastatic potential of LAM cells. A model based on our findings is depicted in Supplementary Fig. S9. In LAM cells, the loss of the TSC2 gene results in hyperactivation of mTOR by Rheb. Activation of mTOR increases protein synthesis and proliferation of LAM cells and inhibits autophagy. Autophagosomes are involved in the elimination of active Src kinase from cells. Autophagy inhibition causes accumulation of phospho-Src(Y416) kinase. Activation of the Src pathway upregulates EMT genes, including Snail and MMP9 and leads to suppression of E-cadherin.

Hyperactivity of Src has been implicated in the development of numerous human cancers and progression to metastases (18). LAM is currently viewed as a low-grade, destructive, metastasizing neoplasm (12, 11). Recent evidence suggests that LAM cells have features similar to cells undergoing EMT. One of the critical steps driving EMT is the repression of E-cadherin, resulting in loss of cell–cell adhesion. E-cadherin is a critical regulator of epithelial junction formation. Dysfunction of the E-cadherin–mediated cell adhesion system plays an important role in tumor progression of the relatively benign tumor to invasive, metastatic carcinoma.

Recent studies have shown that, in cancer cells in which the Src pathway is hyperactive, autophagosomes promote degradation of the active tyrosine Src kinase (17). Autophagy is inhibited in LAM cells due to the mTOR activation (7, 26). In this study, we show the critical role of autophagy in accumulation of active Src kinase in TSC2−/− cells as well as in other models of autophagy-deficient cells. Our results indicate that Src kinase activation promotes migration and invasion of TSC2−/− cells, likely secondary to upregulation of Snail transcription factor, which suppresses E-cadherin expression. Similar role of Src in promoting cell migration and invasion via activation of EMT marker MMP9 has been previously described in breast cancer (32). Increased immunoreactivity for MMPs in lung biopsy specimens from subjects with LAM and TSC2-deficient LAM-like cells was also described (29–31). Such activation of MMP9 plays the critical role for the proteolysis and remodeling of the extracellular matrix that allows cancer cells to invade into the surrounding stroma and promotes metastasis.

Src family kinase inhibitor PP2 was found to enhance the E-cadherin–mediated cell adhesion system, which resulted in the suppression of metastasis of cancer cells (18). Dasatinib and saracatinib are the most clinically studied Src inhibitors (27, 28). Preclinical studies in solid tumor cell lines have shown that both dasatinib and saracatinib consistently inhibit cell

Figure 5. Src inhibition reduces EMT markers in TSC2−/− cells. EEF8 cells were treated for 24 hours with vehicle (DMSO), dasatinib (Dasa; 0.5 μmol/L), saracatinib (Sara; 1 μmol/L), or rapamycin (Rapa; 1μg/mL). A, Snail expression was analyzed by RT-PCR (top) or by immunoblot analysis (bottom), n = 4. B and C, cell lysates were analyzed by immunoblot analysis using antibodies against Src, phospho(Y416)-Src or Snail (B), or MMP9 (C). Graphs, quantification of the immunoblots, n = 3. D, cell lysates were analyzed by zymography of gelatin. Graph, quantification of the zymogram, n = 6. E, EEF8 cells were transfected for 72 hours with Src-specific siRNA or NT siRNA and analyzed by immunoblot analysis. Graph, quantification of the immunoblots, n = 4. F, cell lysates of E were analyzed for MMP9 mRNA using RT-PCR (n = 4). Data, mean ± SD, *P < 0.05; **P < 0.01.
Our findings suggest that the selective inhibition of Src kinase could potentially restore cell adhesion and reduce metastatic tendencies in LAM. Here, we demonstrate that dasatinib and saracatinib significantly decrease migration and invasion ability of TSC2−/− cells. LAM cells exhibit increased activation of the mTOR pathway (33). Recent clinical trials in subjects with TSC or LAM using mTOR inhibitor rapamycin showed that there was a reduction in the size of AMLs and, in some cases, improvement in lung function (9, 34). However, cessation of therapy led to the

Figure 6. Src inhibition attenuates invasiveness of TSC2−/− cells. The invasive properties of EEF4 (A) and EEF4 (C) cells were studied using Matrigel inserts. Serum-deprived cells (5 × 10^4 cells) were loaded in the upper compartment of the chambers. DMSO, rapamycin (1 μg/mL), dasatinib (0.5 μmol/L), or saracatinib (1 μmol/L) was added for 18 hours. Cells on the surface of the Matrigel were visualized by staining with 1% Toluidine blue. Representative images are shown. The invading cells were counted in six random fields (B and D). Data, mean ± SD; n ≥ 3. **P < 0.001. Scale bar, 100 μm.
regrowth of tumors and diminished pulmonary functions (9, 10, 35). In our study, rapamycin had no effect on the migration and invasion activity of TSC2−/− cells. These data are of particular clinical relevance because circulating LAM cells were found in the blood and plural fluid of women with LAM and these cells might be the source for invasion of LAM cells into the lungs (13). Thus, failure of rapamycin to affect cell migration or invasion of TSC2−/− cells may explain the transient nature of rapamycin efficacy in LAM. Our study suggests that the selective inhibition of Src kinase could potentially restore cell adhesion and reduce metastatic tendencies of TSC2−/− cells in LAM. Saracatinib treatment notably decreased lung colonization of TSC2−/− cells in vivo. Rapamycin either alone or in combination with saracatinib did not provide additional benefits.

Taken together, our findings highlight a role of Src kinase in the pathogenesis of LAM. Our data demonstrate that activated Src kinase promotes EMT in TSC2−/− cells and increases their metastatic potential. Src kinase inhibitors dasatinib and saracatinib notably decreased lung colonization of TSC2−/− cells in vivo. Rapamycin either alone or in combination with saracatinib did not provide additional benefits.

Figure 7. Effect of Src inhibition on lung colonization of EEF cells in vivo. EEF8-Luciferase cells were treated for 18 hours with DMSO, rapamycin (1 μg/mL), saracatinib (1 μmol/L), or by both rapamycin and saracatinib (A–D). EEF4-Luciferase cells were treated for 18 hours with DMSO or saracatinib (1 μmol/L; E–H). Cells were then injected intravenously into female CB17 SCID mice and after 6 hours, lung colonization was measured using bioluminescence. Representative images are shown (A and E). Total photon flux per second present in the chest region after injection of EEF cells is expressed as a percentage of DMSO-treated EEF cells (B and F). Lungs were dissected 24 hours after cell injection and bioluminescence was imaged in Petri dish (C and G). Total photon flux per second present in the dissected lungs after injection of EEF cells is expressed as a percentage of DMSO-treated EEF cells (D and H). Data, mean ± SD; n ≥ 3. *, P < 0.05; **, P < 0.001 compared with DMSO-treated cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Tyryshkin, N.T. Eissa
Development of methodology: A. Bhattacharya, N.T. Eissa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Bhattacharya
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Tyryshkin, N.T. Eissa

2004 Cancer Res; 74(7) April 1, 2014 Cancer Research
Writing, review, and/or revision of the manuscript: A. Tyryshkin, A. Bhattacharya, N.T. Eissa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N.T. Eissa
Study supervision: N.T. Eissa

Acknowledgments

The authors thank Dr. Raymond Yeung of the University of Washington (Seattle, WA) for kindly providing Eker rat embryos fibroblasts TSC2−/− cells and wild-type controls. The vector encoding cSrc cDNA was a gift from Dr. Wouter Moolenaar.

References


Grant Support

This study was supported by funds from National Heart, Lung, and Blood Institute (R01 HL69033) and from the NIH Common Fund, through the Office of Strategic Coordination/Office of the NIH Director, and the National Center for Advancing Translational Sciences (U54 TR000961).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 2, 2013; revised January 9, 2014; accepted January 14, 2014; published online April 1, 2014.

Src Kinase Is a Target in Lymphangioleiomyomatosis

www.aacrjournals.org Cancer Res; 74(7) April 1, 2014
Src Kinase Is a Novel Therapeutic Target in Lymphangioleiomyomatosis

Alexey Tyryshkin, Abhisek Bhattacharya and N. Tony Eissa


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/74/7/1996

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/07/21/74.7.1996.DC1

Cited articles
This article cites 35 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/74/7/1996.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/74/7/1996.full.html#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.