Published in final edited form as:

Cancer Res. 2014 April 1; 74(7): 1996–2005. doi:10.1158/0008-5472.CAN-13-1256.

Src Kinase is a Novel Therapeutic Target in Lymphangioleiomyomatosis

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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 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. Further, 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.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutation in either the tuberous sclerosis complex 1 (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 30–40% 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 mammalian target of rapamycin (mTOR) is a serine/threonine kinase that positively regulates cell growth, proliferation, and survival (5). TSC2 is a negative regulator of the mTOR complex 1 (mTORC1) (6,7). Therefore, hyper-activation of mTORC1 and inhibition of autophagy are observed in TSC2^{-/-} LAM cells (8). However, many of the clinical and pathological features of LAM remain unexplained by our current understanding of the function of these genes. Activation of mTORC1 is sensitive to inhibition by rapamycin, which has been used in the treatment of LAM (9,10). Rapamycin treatment improved pulmonary functions and reduced the size of angiomyolipoma (AML) in TSC and LAM subjects. Unfortunately, cessation of rapamycin therapy was followed by regrowth of tumors and the decline of pulmonary functions (9,10). Accordingly, alternative or

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“The authors disclose no potential conflicts of interest.”

combinational therapies are needed to treat LAM. Identification of novel therapeutic targets, other than mTOR, might allow such therapy.

Accumulating evidence supports the hypothesis that LAM is a low-grade, destructive, metastasizing neoplasm (12,13). LAM cells are found in blood, urine, and chylous fluids of LAM subjects with AML (11). If the metastatic hypothesis for LAM is correct, then AML or renal tumors might be the source. Consistent with this notion, the morphology and immunohistochemical characteristics of AML and LAM cells are very similar. However, not all subjects with LAM have detectable AML, and the uterus has also been proposed as a potential source (12,13).

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) (14). Src family kinases are non-receptor 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(Tyr416), pStat3(Tyr705), Stat3, pErk1/2(Thr202/Tyr204), Erk1/2, S6, pS6(Ser235/236), pFAK(Tyr925), pFAK(Tyr397), mTOR, U0126 (all from Cell Signaling), tuberlin, rabbit E-cadherin, MMP9, Snail (all from Santa Cruz), mouse E-cadherin (BD), Src (Millipore), pSrc(Tyr418) (LifeSpan Biosciences) and HMB45 (Enzo Life Sciences). Src kinase inhibitors PP2 and SU6656 were purchased from Calbiochem. Rapamycin, dasatinib and saracatinib were purchased from LC Laboratories.

Cell culture and tissue samples

Eker rat embryonic fibroblasts EEF4 (TSC2^{+/+}) and EEF8 (TSC2^{-/-}) were maintained in Dulbecco's modified Eagle's medium (DMEM)/ F12 mixture (1:1) containing 10% heat-inactivated FBS. Lung tissues of normals and of subjects with LAM were obtained from 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-min incubation at room temperature with Alexa fluor 594-labeled secondary antibodies. Coverslips were mounted by SlowFade gold antifade reagent with 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 min at room temperature. Slides were developed using Vectastatin Elite ABC kit (Vector Labs) and counterstained with hematoxylin. Images were viewed using a Zeiss Axiovert 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 μ L), washed with medium to remove floated and detached cells. Wound areas were photographed (magnification 100x) at the start and 18 hr after treatment to assess the degree of wound closure. Data are expressed in $\mu\text{m}^2 \times 1000$.

Cell invasion assay

Cells were studied using Matrigel inserts (BD Biosciences). Serum-deprived cells (5×10^4 cells) were loaded in the upper compartment of the chambers and the bottom wells were filled with chemo-attractant (complete media with 10% FBS). After incubation for 18 hr, the membranes were processed and the non-migrating 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 6 random fields under a microscope.

Real-Time PCR

RNAs were purified using Rneasy Mini Kit (Qiagen) and cDNA synthesis was performed using 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 PerfeCTa™ qPCR 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, 6–8 weeks of age, were purchased from Jackson Laboratory. Cells were transfected with pGL4.51[*luc2*/CMV/Neo] vector (Promega), expressing luciferase, using Lipofectamine 2000 (Invitrogen). For intravenous injections, 1×10^6 cells were injected into the retro-orbital vein. Ten minutes prior to imaging, animals were injected with Luciferin (Promega; 120mg/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.

Statistical analysis

All blots and data are representative of at least three independent experiments. The Student's 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) (12, 21). Phosphorylation of the ribosomal protein S6 was increased in LAM lung tissues compared to 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 (19) in LAM lungs (Fig. 1C). Importantly, we found increased phosphorylation of Src on Tyr416 in LAM lung tissues compared to normal lungs (Fig. 1D). These findings were further confirmed by analyzing human LAM lungs by immunofluorescence (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 (21). Phosphorylation of Tyr416, in the activation loop of the kinase domain, upregulates Src kinase activity (35). 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 signal transducer and activator of transcription 3 (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 (20).

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 (Fig. S2). These data confirm that LAM cells have increased Src and STAT3 activities.

Src and STAT3 are activated in TSC2^{-/-} cells

We studied Eker rat embryonic fibroblasts (EEF) TSC2^{+/+} wild-type (EEF4) and TSC2^{-/-} mutant cells (EEF8). These cells are well characterized as a cellular model for LAM and TSC (14,22). EEF8 cells (TSC2^{-/-}) did not express Tuberin, had increased activity of mTOR and suppressed autophagy (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 if TSC2^{-/-} cells have increased Src activity. We found that phosphorylation of Src was increased in the TSC2^{-/-} cells (Fig. 2A and 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, small interfering RNA (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 (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 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–B). Moreover, we found overphosphorylation of FAK on Y397 and Y925 sites in EEF8 cells (Fig. 3C–D). FAK-Y397 autophosphorylation plays an important role in FAK binding to Src kinase and forming of active FAK-Src complex (23). Recruitment of Src kinase results in phosphorylation of FAK-Y925 and triggers a Ras-dependant activation of MAP kinase pathway (24). To evaluate MAP kinase pathway activation in EEF8 cells, we examined phosphorylation of Erk. We found increased level of phosphorylated Erk in EEF8 cells, compared to 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, 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 TSC2 gene leads to mTOR activation and autophagy inhibition (25). 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 (Fig. S6 A–B). Furthermore, mouse embryonic fibroblasts (MEF) derived from *Atg7*^{-/-} mice and *Atg5*^{-/-} mice had increased active Src (Fig. S6 C–D). Finally, treatment of wild-type EEF4 cells with autophagy-lysosome pathway inhibitor chloroquine resulted in increased active Src (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 (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–B). In wild-type cells (EEF4), E-cadherin was readily detectable and localized predominantly at the plasma membrane, where 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 co-localize 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). Further, the increase in Snail expression was limited to LAM cells identified by positive staining for HMB45 (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 (26, 27). Both inhibitors reduced levels of Snail, whereas rapamycin had no effect (Fig. 5A–B). Src inhibition also reduced levels of MMP9, as determined by immunoblot and further confirmed by gelatin zymogram (Fig. 5C–D). Overall, dasatinib and saracatinib appeared 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–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 (32–34) 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 (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 to control cells and that Src inhibitors significantly decreased the proliferation of EEF8 cells (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 hr, the membranes were processed and the invading cells were counted in 6 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–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. Further, there was no effect of Src inhibitors on the invasiveness in the EEF4 cells, probably because of low invasiveness of these cells (Fig. 6C–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 pre-treated with vehicle (DMSO), rapamycin (1 µg/ml), saracatinib (1 µM) or both rapamycin and saracatinib. 1x10⁶ cells were then intravenously injected into female CB17 SCID mice. Six hours following injection of cells, and 10 minutes prior to imaging, animals were injected intraperitoneally with 120 mg/kg, Luciferin. Bioluminescent signals were recorded using Xenogen *in vivo*

imaging system (Fig. 7A–B). Total photon flux at the chest region was analyzed. At 24 hr time point after cell injection, mice were sacrificed and their lungs were dissected and imaged in Petri dish (Fig. 7C–D). Saracatinib significantly reduced the number of EEF8-Luc cells that was detected in the lungs at 6 and at 24 hr post injection. Rapamycin treatment had no significant effect. Further, 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 hr but not after 6 hr of the cell injection (Fig. 7E–H). Further, although the decrease in lung colonization was significant at 24 hr 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 panels D and H in figure 7. Thus, the consequences of Src inhibition were more pronounced in TSC2^{−/−} cells compared to control cells. These results suggest that Src inhibition can reduce the metastatic potential for TSC2^{−/−} cells

Discussion

This study has three major novel findings. The first is that Src is activated in LAM cells. The second is that Src activation contributes to the pathogenesis of LAM by promoting EMT in TSC2^{−/−} LAM cells. The third is that Src inhibition can attenuate the oncogenic and metastatic potential of LAM cells. A model based on our findings is depicted in figure S9. In LAM cells, the loss of 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 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 (12,13). 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 (7,25). 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 (28). Increased immunoreactivity for MMPs in lung biopsy specimens from subjects with LAM and TSC2-deficient LAM-like cells were also described (32–34). 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 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 (26,27). Preclinical studies in solid tumor cell lines have shown that both dasatinib and saracatinib consistently inhibit cell

proliferation. 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 (29). Recent clinical trials in subjects with TSC or LAM using mTOR inhibitor rapamycin showed that there was a reduction in the size of angiomyolipomas and, in some cases, improvement in lung function (9,30). However, cessation of therapy led to the regrowth of tumors and diminished pulmonary functions (9,10,31). 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 (11). 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 decrease migration and invasion ability of TSC2^{-/-} cells. These results will be valuable for understanding the nature of EMT in LAM cells and provide a novel therapeutic target to prevent LAM cell dissemination. The efficacy of dasatinib and saracatinib, used as a single agent or in combination with mTOR inhibitors might improve treatment outcomes in LAM. The use of multiple drug therapy has the advantage of reducing the dose of each drug and thus can minimize side effect. Overall, our study establishes Src as a novel therapeutic target in LAM and provide encouragement for further preclinical and clinical studies of the use of Src inhibitors. Several Src inhibitor are already being tested in clinical trials, which can enhance translation of our findings to the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Eker rat embryos fibroblasts TSC2^{-/-} cells and wild-type controls were kindly provided by Dr. Raymond Yeung, University of Washington. The vector encoding cSrc cDNA was a gift from Dr. Wouter Moolenaar. 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 (UH2 TR000961)

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Figure 1. Sections of normal and LAM lungs were stained with LAM cell marker HMB45 antibody (A), or lysed and analyzed by immunoblot using antibodies against S6 and phospho-S6 (B), p62 (C), Src kinase and phospho-Src (Y416) kinase (D), or STAT3 and phospho-STAT3 (Y705) (E). Blotting with β -actin antibody was used as a loading control. Scale bar, 100 μ m.

Figure 2. Activation of Src and STAT3 in TSC2^{-/-} cells (EEF8)

(A–B) Cell lysates of EEF4 and EEF8 were subjected to immunoblot 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 4',6-diamidino-2-phenylindol dihydrochloride (DAPI) to visualize nuclei (blue). Graph indicates 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 μ M), or SU6656 (Su; 10 μ M) for 4 hr prior to lysis. Cell lysates were subjected to immunoblot analysis. (E) HeLa cells were transfected for 72 hr with control non-target (NT) siRNA or TSC2-specific siRNA. Cells were lysed and immunoblot was done. Graphs indicate quantification of phospho-Src and phospho-STAT3, normalized to total Src or STAT3 expression, respectively, n=3. (F) EEF4 cells were transfected for 24 hr with Src kinase or LacZ control plasmids. Cell lysates were analyzed, compared to EEF8, by immunoblotting. Blotting with β -actin antibody was used as a control. Data are mean $\pm$ SD, *P<0.05, **P<0.001. Scale bar, 10 μ m

Figure 3. Src kinase mediates phosphorylation of focal adhesive kinase (FAK) and activation of MAPK pathway in TSC2^{-/-} cells

(A–E) EEF4 and EEF8 cells were analyzed by Real time PCR for FAK mRNA (A) or by immunoblot using antibodies against FAK (B) phospho-FAK(Y397) (C) phospho-FAK(Y925) (D) or Erk and phospho-Erk(Thr202/Tyr204) (E). In B, C and E, graphs indicate quantification of the immunoblots, n=6, 3 or 4, respectively. Protein expression was normalized to β -actin expression, and then to EEF4 group. Phospho-Erk was normalized to total Erk, and then to EEF4 group. (F) EEF4 cells were transfected for 24 hr with a plasmid encoding Src kinase or LacZ as a control and analyzed by immunoblot. Graph indicates quantification of the immunoblots. Phospho-Erk was normalized to total Erk expression, n=3. (G) EEF8 cells were transfected with Src specific siRNA or control (NT) siRNA for 72 hr and analyzed by immunoblot. Graph indicates the quantification of the immunoblots, n=4. (H) EEF8 cells were treated for 4 hr with either DMSO or Src inhibitors PP2 (25 μ M), SU6656 (10 μ M), dasatinib (0.5 μ M) or Saracatinib (1 μ M) and then analyzed by immunoblot. Blotting with actin antibody was used as a control. Data represent mean $\pm$ SD, n = 3, **P<0.001.

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 real time PCR (graphs in **C** and **F**) to evaluate E-cadherin (**A–B**), Snail (**C–E**), or MMP9 (**F**). In **B** and **D**, cells were stained with DAPI to visualize nuclei (blue). In (**G**), EEF4 cells were transfected for 16 hr with Src or LacZ control vector and analyzed by immunoblot. β -actin antibody was used as a loading control. Graphs in **G** indicate the quantification of the immunoblots. Data represent mean $\pm$ SD, n 3. ** $P < 0.001$. Scale bar, 10 μ m.

Figure 5. Src inhibition reduces EMT markers in TSC2^{-/-} cells

EEF8 cells were treated for 24 hr with vehicle (DMSO), dasatinib (Dasa; 0.5 μ M), saracatinib (Sara; 1 μ M) or rapamycin (Rapa; 1 μ g/ml). (A) Snail expression was analyzed by real time PCR (upper panel) or by immunoblot (lower panel), n=4. (B–C) Cell lysates were analyzed by immunoblot using antibodies against Src, phospho(Y416)-Src or Snail (B), or MMP9 (C). Graphs indicate the quantification of the immunoblots, n=3. (D) Cell lysates were analyzed by zymogram of Gelatin. Graph indicates the quantification of the zymogram, n=6. (E) EEF8 cells were transfected for 72 hr with Src-specific siRNA or non-target (NT) siRNA and analyzed by immunoblot. Graph indicates the quantification of the immunoblots, n=4. (F) Cell lysates of (E) were analyzed for MMP9 mRNA using real time PCR (n=4). Data are mean $\pm$ SD, *p < 0.05.

Figure 6. Src inhibition attenuates invasiveness of TSC2^{-/-} cells

The invasive properties of EEF8 (**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 ($1 \mu\text{g/ml}$), dasatinib ($0.5 \mu\text{M}$) or saracatinib ($1 \mu\text{M}$) was added for 18 hr. 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 6 random fields (**B**, **D**). Data are expressed as mean $\pm$ SD, $n = 3$. $**P < 0.001$. Scale bar, $100 \mu\text{m}$.

Figure 7. Effect of Src inhibition on lung colonization of EEF cells *in vivo*

EEF8-Luciferase cells were treated for 18 hr with DMSO, rapamycin (1 μ g/ml), saracatinib (1 μ M) or by both rapamycin and saracatinib (A–D). EEF4-Luciferase cells were treated for 18 hr with DMSO or saracatinib (1 μ M) (E–H). Cells were then injected intravenously into female CB17 SCID mice and after 6 hr lung colonization was measured using bioluminescence. Representative images are shown (A, E). Total photon flux/second present in the chest region after injection of EEF cells is expressed as a percentage of DMSO treated EEF cells (B, F). Lungs were dissected 24 hr post cell injection and bioluminescence was imaged in Petri dish (C, G). Total photon flux/second present in the dissected lungs after injection of EEF cells is expressed as a percentage of DMSO treated EEF cells (D, H). Data are mean $\pm$ SD, n = 3. *P<0.05, **P<0.001 compared to DMSO treated cells.