TSC2 modulates cell adhesion and migration via integrin-α1β1

Lyn M. Moir,1,2 Judith L. Black,1,2 and Vera P. Krymskaya3,4
1Cell Biology, Woolcock Institute of Medical Research, Sydney 2Discipline of Pharmacology, University of Sydney, Sydney, New South Wales, Australia; 3Department of Medicine and 4Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania

Submitted 9 January 2012; accepted in final form 22 August 2012

Moir LM, Black JL, Krymskaya VP. TSC2 modulates cell adhesion and migration via integrin-α1β1. Am J Physiol Lung Cell Mol Physiol 303: L703–L710, 2012. First published August 24, 2012; doi:10.1152/ajplung.00414.2011.—Recent evidence suggests that the rare and progressive lung disease lymphangioleiomyomatosis (LAM) is metastatic in nature. Dysfunction of the tumor suppressor genes tuberous sclerosis complex (TSC), in particular mutational inactivation of TSC2, enhances both cell proliferation and migration. Although substantial progress has been made in understanding the role of TSC2 in abnormal LAM cell proliferation and its pharmacological targeting, the mechanisms underlying the enhanced migratory capacity in LAM are not well understood. In this study, we examined the role of TSC2 in cell attachment, spreading, and migration, processes that contribute to the metastatic phenotype. Here we show that loss of TSC2 increased both the attachment and spreading of mouse embryonic fibroblasts to the extracellular matrix proteins collagen type I and fibronectin and that reexpression of TSC2 reduced these effects. Integrin-α1β1 modulated cell migration with the β1-subunit involved in cell attachment and spreading as shown by using functional blocking antibodies. Loss of TSC2 increased integrin-α1 expression, and inhibition of this integrin subunit reduced cell migration. The enhanced attachment and spreading were independent of the intracellular signaling pathways mammalian target of rapamycin complex 1 and Rho-associated kinase, as pharmacological inhibition with rapamycin or Y27632, respectively, was without effect. Together, these data demonstrate that TSC2 controls cell migration, attachment, and spreading through the α1β1-integrin receptor and thus suggest a potential therapeutic target for the treatment of increased cell invasiveness in LAM.

PULMONARY LYMPHANGIOLEIOMYOMATOSIS

Pulmonary lymphangioleiomyomatosis (LAM) is a progressive and usually fatal rare lung disease affecting predominantly women of childbearing age (20, 35). LAM manifests as widespread neoplastic lesions of smooth muscle-like LAM cells, which lead to the cystic destruction of the lung and loss of pulmonary function. LAM occurs sporadically or manifests in association with tuberous sclerosis complex (TSC) due to mutational inactivation of the tumor suppressor genes tuberous sclerosis complex 1 (TSC1) and TSC2 (2, 33). Mutations in the TSC2 gene arise more frequently and exhibit a more severe disease phenotype compared with the milder phenotype induced by TSC1-disease associated mutations (32).

Although LAM was originally considered benign, recent evidence suggests that it is a metastatic disease. Tumors of the kidney, angiomylipomas (AMLs), occur in 30–50% of sporadic LAM and in most patients with TSC-LAM. In addition, circulating LAM cells with TSC2 loss of heterozygosity have been found in blood, urine, and chylous fluid from patients with LAM, suggesting the potential for metastatic dissemination of LAM cells (3). Furthermore, identical TSC2 mutations have been found in LAM cells in the lungs and AML cells in renal tumors of TSC patients with LAM, suggesting that these cells have a common origin (38). Moreover, LAM nodule recurrence was observed after a single-lung transplantation in a patient with LAM, suggesting that LAM cells can migrate abnormally and metastasize in vivo (21). Taken together, this suggests that loss of TSC2 function may contribute to the metastatic dissemination in LAM. The cellular and molecular mechanisms of LAM cell metastasis, however, are not fully understood.

Metastatic cell dissemination involves two separate and distinct processes: enhanced cell proliferation and cell migration. Our previous studies linked mutational inactivation of TSC2 in LAM to the constitutive activation of p 70 S6 kinase (S6K1) and abnormal LAM cell growth (16). TSC2 has been shown to regulate cell growth and proliferation through its role as a negative regulator of Rheb and mammalian target of rapamycin (mTOR)/S6K1 signaling. Several studies show that TSC2 dysfunction promotes cell motility and tumorigenesis; for example, TSC2-null rat embryonic fibroblasts show anchorage-independent growth and colony formation in soft agar (34). In addition, TSC2-null ELT3 cells derived from Eker rat uterine leiomyomas develop tumors in nude mice (39). Our published studies show that primary cultures of human LAM-derived cells have increased migratory activity and invasiveness that are abrogated by TSC2 expression (15). Collectively, these studies demonstrate that TSC2 plays a role in cell motility; however, the precise cellular mechanisms of TSC2-dependent regulation of cell motility remain unknown.

Cell-extracellular matrix (ECM) interaction regulates many cellular processes, including cell proliferation, migration, and invasion. Therefore, any change in ECM deposition could influence cellular function. The composition of the ECM is altered in LAM; for example, collagen is increased in LAM lungs (28), and fibronectin is present in LAM cell foci (5). Thus these changes may modulate cell function. Cells interact with the ECM through receptors present on their cell surface, including integrins, which are heterodimeric glycoproteins consisting of an α- and β-subunit (19). Each of these receptors binds to ECM proteins with varying affinity; for example, integrin-α5β1 binds strongly to fibronectin, whereas integrin-α1β1 and -α2β1 are known collagen receptors. Integrins-α1β1 and -α2β1 have been implicated in the regulation of cell growth and migration in other diseases (24). However, little is known about the adhesion process in LAM. Recent evidence has implicated integrins in the adherence and migration of lymphatic endothelial cells in tumor-
induced lymphangiogenesis, thus indicating a role for integrins in tumor metastasis (8, 9).

In this study, we examined the motility and adhesion of currently available TSC2-null cellular models of LAM and their wild-type controls. We investigated whether cell-matrix interactions are modulated by TSC2 loss and the roles of the mTORC1 pathway and integrin receptors.

MATERIALS AND METHODS

Cell culture. TSC2-null cells derived from Eker rat uterine leiomyoma (ELT3) cells were cultured as previously described (10, 12, 18). A littermate-derived pair of TSC2−/− mouse embryonic fibroblasts (MEFs) and TSC2+/+ MEFs, both of which were also null for p53 for immortalization, and isogenic 323-TSC2-null MEFs expressing endogenous p53 and their stable clones reexpressing TSC2 (323-TSC2+ MEFs) were cultured in DMEM containing penicillin-streptomycin and 10% FBS (14). For experiments cells were seeded at a density of 0.9–1.75 × 10^4 cells/cm² and cultured in medium containing 0.5% serum for 24–48 h before the assay as indicated.

Migration assay. Migration was assayed using Transwell insert techniques as previously described (13). Briefly, cells (200,000 cells/well) were placed into serum-reduced (0.5% FBS) medium in the upper chamber of the 8-μm-pore Transwell insert precoated with collagen type I (10 μg/ml). Cells were allowed to migrate for 4 h in an incubator at 37°C/5% CO₂. Nonmigrated cells were scraped off the upper side, and migrated cells on the lower surface were fixed, stained, and counted. Where indicated, cells were preincubated with a monoclonal function blocking antibody to specific integrin subunits for 30 min before the migration assay.

Surface coating with ECM proteins. Rat plasma fibronectin, rat tail collagen type I, and mouse laminin (purified from Engelbreth-Holm/Swarm sarcoma) were diluted in sterile PBS to concentrations from 0.1–10 μg/ml. Diluted ECM proteins were adsorbed onto 96-well, 24-well, 10-cm tissue culture plates or migration inserts overnight at 4°C. Unoccupied protein binding sites were blocked by incubation with 1% BSA for 30 min, after which the surface was washed twice with sterile PBS before the addition of the cell suspension.

Cell attachment assay. Ninety-six-well plates were precoated with ECM proteins as described above. Cells were trypsinized, and 5 × 10^4 cells/cm² were seeded for 2 h at 37°C/5% CO₂. Unattached cells were discarded, and the attached cells were washed twice with PBS. Cells were then fixed with 1% formaldehyde (methanol free) for 5 min and stained with 0.5% toluidine blue solution for a further 5 min. Stained cells were then washed with distilled water and solubilized with 1% SDS solution. The relative number of attached cells was measured using spectrophotometry at an absorbance of 595 nm. Where indicated, the cells were incubated with the mTORC1 inhibitor rapamycin (2–200 nM), the Rho-associated kinase (ROCK) inhibitor Y27632 (0.3–30.0 μM), or function blocking monoclonal anti-integrin antibodies, anti-α1 (Clone Ha31/8; BD Pharmingen), anti-α5 (Clone 5H10 –27 MFR5; Biolegend), and anti-β1 (Clone Ha2/5; BD Pharmingen) (each 10 μg/ml) for 30 min before addition of the cells to the wells.

Cell-spreading assay. Cells were seeded in 96-well plates, precoated with collagen type I for 2 h at 37°C/5% CO₂. Unattached cells
were discarded, and the attached cells were washed with PBS. Cells were then fixed with 1% formaldehyde (methanol free) for 5 min and then imaged using a Nikon microscope. Cell spreading was measured by manually counting the number of cells in a defined area, which showed protrusions (4). Cells that were not spread were round in morphology, whereas spread cells were counted as cells that displayed any clear form of protrusion.

Flow cytometry. Cell surface expression of the integrin subunits-\(\alpha_1\), -\(\alpha_2\), -\(\alpha_5\), and -\(\beta_1\) was determined using flow cytometry as previously described (1, 29). Cells were harvested using trypsin-EDTA (0.025% wt/vol) for 3 min, incubated with 1% BSA in PBS for 15 min, and then washed with 0.1% BSA in PBS. Cells were incubated with the specific anti-integrin antibody (anti-\(\alpha_1\), Clone Ha31/8; BD Pharmingen), anti-\(\alpha_2\) (Clone Ha1/29; BD Pharmingen), anti-\(\alpha_5\) (Clone 5H10–27 MFR5; Biolegend), and anti-\(\beta_1\) (Clone Ha2/5; BD Pharmingen) or appropriate isotype control (1 \(\mu\)g/ml) for 30 min on ice. Following wash with 0.1% BSA, cells were incubated with anti-hamster-phycocerythrin-conjugated secondary antibody (Jackson Immunotech) in the dark, for 30 min on ice. Next, the cells were washed with PBS and fixed in 4% formaldehyde (methanol free) for 15 min before analysis on a FACS Calibur flow cytometer (Becton Dickinson). Typically 10,000 events were collected in the gated region of the forward scatter/side scatter plots to examine cell surface expression of integrin subunits. Data were analyzed using FlowJo software (TreeStar).

Statistical analysis. Data are expressed as mean values \(\pm\) SE. Data were analyzed using a \(t\)-test or one-way ANOVA with Bonferroni correction post hoc where appropriate. A probability value of less than or equal to 0.05 was considered statistically significant.

Fig. 3. Loss of TSC2 increases spreading of attached cells. Cells were allowed to attach to and spread on collagen type I for 2 h before fixation. Images were taken, and the number of spread cells relative to the number of attached cells was calculated (\(n = 3\)). *\(P < 0.05\) compared with TSC2+/+ cells, using unpaired \(t\)-test.

Fig. 4. Cellular attachment to ECM proteins is independent of mammalian target of rapamycin complex (mTORC1) and Rho-associated kinase (ROCK) signaling pathways. Cells were pretreated with rapamycin and Y27632 for 30 min before the attachment assay. Inhibition of mTORC1 using rapamycin had no effect on the attachment of MEFs (\(n = 4\)) (A), 323-MEFs (\(n = 3\)) (B), or ELT3 cells (\(n = 3\)) (C) to collagen type I. Similarly, treatment with the ROCK inhibitor Y27632 had no effect on the attachment of MEFs (\(n = 3\)) (D), 323-MEFs (\(n = 4\)) (E), or ELT3 cells (\(n = 3\)) (F).
RESULTS

**TSC2 inhibits cell migration.** Since our previous data demonstrate that TSC2 inhibits the increased migration and invasiveness of LAM-derived smooth muscle-like cells (15), we examined the migratory activity of TSC2+/+ and TSC2−/− MEFs. As seen in Fig. 1, loss of TSC2 significantly promotes migration in this cell type, as migration of serum-reduced TSC2−/− MEFs through a collagen type I-saturated membrane was markedly increased compared with TSC2+/+ cells. These data further support the evidence that loss of TSC2 induces cell migration and suggest that TSC2 dysfunction contributes to neoplastic transformation in LAM.

**Cell attachment is modulated by ECM proteins, and loss of TSC2 enhances this attachment.** In order for cells to migrate they must attach and spread; therefore, we first examined the attachment of wild-type and TSC2-null cells to the ECM proteins fibronectin, collagen type I, and laminin and compared this to attachment to tissue culture plastic. The efficacy of wild-type MEFs (TSC2+/+) to attach to the ECM proteins was fibronectin (FN) and collagen type I (Col I) > laminin (LN) (Fig. 2A). Dysfunction of TSC2 further modulated cell attachment as shown in Fig. 2, A and B. Thus attachment of TSC2−/− MEFs was greater than TSC2+/+ MEFs to FN, Col, and LN as demonstrated using the toluidine blue attachment assay (Fig. 2A) and manual cell counts (Fig. 2B). However, these MEFs were also null for p53; thus we needed to confirm that these results were not due to a lack of p53. Using 323-TSC2-null MEFs, which endogenously express p53, and their stable clones expressing both TSC2 and p53, we confirmed that attachment was modulated by both ECM proteins and TSC2, as reexpression of TSC2 reduced attachment to FN and Col I (Fig. 2C). Furthermore, attachment of ELT3 cells (which are TSC2 null and a well-established cellular model for LAM; 6, 10, 12) was also dependent on ECM proteins, with increased attachment to fibronectin and collagen type I compared with tissue culture plastic (Fig. 2D). These data demonstrate that TSC2 loss enhances cell attachment to FN and Col I and suggest that TSC2-dependent modulation of cell interaction with the ECM may contribute to increased cell motility in LAM.

**TSC2 dysfunction increases cell spreading.** Since migration requires, not only cell attachment, but also cell spreading, we next examined whether loss of TSC2 also altered the ability of the cells to spread on collagen type I. As seen in Fig. 3, the majority of TSC2+/+ MEFs have a round shape with a few lamellipodia protrusions, indicating the initial stages of spreading. In contrast, at the same time point, the majority of TSC2−/− MEFs show either a flattened round-like shape or cells with typical fibroblast-like extended morphology. Thus cells deficient for TSC2 show, not only increasing attachment, but also spreading characteristic of neoplastic cells.

**Cell attachment is not modulated through mTORC1 or ROCK signaling pathways.** Since TSC2 regulates the mTORC1 pathway, we examined, using the specific inhibitor rapamycin, whether the enhanced attachment and spreading in TSC2-null cells is regulated via mTORC1. We found that rapamycin (2–200 nM) had little effect on the attachment of MEFs (Fig. 4A), 323-MEFs (Fig. 4B), or TSC2-null ELT3 cells (Fig. 4C) to collagen type I (Fig. 4, A–C) or fibronectin (data not shown). TSC2 also regulates the RhoA GTPase pathway (10, 15). However, treatment with the Rho-associated protein kinase p160 ROCK inhibitor Y27632 had no effect on the attachment of MEFs (Fig. 4D), 323-MEFs, or ELT3 cells (Fig. 4, E and F) to collagen type I. Similarly, inhibition of Rho-associated kinase II (ROCK II) using HA-1077 had no effect on ELT3 cell attachment to collagen type I (data not shown). Thus increased attachment of TSC2-null cells is independent of mTORC1 and ROCK signaling.

**Cell attachment, spreading, and migration is modulated by integrin-α1β1.** Integrins are a family of cell adhesion molecules that bind to ECM proteins and facilitate cell attachment and spreading. Therefore, we examined the expression of integrin receptors-α1 (Fig. 5A), α2, α5 (data not shown), and β1 (Fig. 5B) on the surface of MEFs using flow cytometry. Both TSC2+/+ and TSC2−/− MEFs express the integrin subunit-β1 (Fig. 5, A and C). The cell surface expression of the integrin subunit-α2 was also increased on TSC2−/− MEFs; however, its expression was very low, and therefore we did not continue to examine this receptor (data not shown). Furthermore, although both TSC2+/+ and TSC2−/− MEFs expressed the integrin subunit-α5, expression levels were the same between cell types (data not shown).

We also examined the outcomes of blocking α1-, α5-, and β1-integrin subunits on cell attachment using functional blocking antibodies against α1-, α5-, and β1-integrins. We found that blocking the β1-integrin subunit decreased attachment of MEFs (Fig. 6A, n = 6), 323-MEFs (Fig. 6C), and ELT3 cells (Fig. 6D) to collagen type I. As seen in representative images...
in Fig. 6, TSC2-null ELT3 cells treated with a β1-function blocking antibody show a round morphology (Fig. 6D, right), affecting, not only attachment, but also spreading compared with cells treated with control IgM (Fig. 6C, left). As shown in Fig. 6E, blocking integrin-β1 also reduced the spreading of attached cells, with the cells remaining round in shape. Interestingly, blocking integrin subunit-α1 alone (Fig. 6A, n = 9) or -α5 alone (Fig. 6B, n = 6) had no effect on the attachment of MEFs to collagen type I when compared with the isotype control. Furthermore, blocking α1β1 (Fig. 6A, n = 5) or α5β1 (n = 1; data not shown) did not enhance the inhibitory effect of blocking β1 alone, suggesting that attachment and spreading involves the β1-subunit alone.

**Integrin-α1 is involved in TSC2-null cell migration.** Since TSC2-null cells migrate more than TSC2+/+ cells, we examined the role of the integrins-α1 and -β1 in migration. As shown in Fig. 7, blocking integrin-α1 alone or in combination with -β1, but not -β1 alone, significantly inhibited the enhanced migration of TSC2−/− MEFs. These data suggest that increased migration of TSC2−/− MEFs is modulated by α1β1-integrins.

**DISCUSSION**

Neoplastic transformation in pulmonary LAM is not well understood. Cellular metastasis is not a single event but requires a series of steps that enable cells to invade. These include cell attachment, spreading, and migration. Our findings show that loss of TSC2 promotes cellular attachment, spreading, and migration that these enhanced metastatic properties are modulated by the β1-family of integrins, in particular α1β1. In addition, we have shown that this enhanced attachment is independent of intracellular signaling through mTORC1 and ROCK.
In the present study, we demonstrate that ECM proteins modulate the fibronectin deposition. Collagen is increased in LAM lungs and this structure is disrupted in LAM, with altered collagen and ROCK inhibition, at concentrations known to inhibit targeting, suggesting that changes in ECM composition may perpetuate the disease progression.

Adhesion of a cell to its surrounding environment is mediated by cell surface receptors, which couple the ECM outside a cell to the actin cytoskeleton inside the cell. A cycle of attachment and detachment of cells to the ECM via integrin receptors is thought to be important for cell migration. Altered cell-ECM interactions are implicated in the invasion and metastasis of tumor cells. The β1-family of integrins plays a role in metastasis in many diseases including gastric, ovarian, and lung cancers. In the present study, we identified differences in the expression of the collagen receptors α1β1 and α2β1 on TSC2-null cells and wild-type controls. TSC2-null cells expressed higher levels of the integrin subunits-α1 and -β2, but both ubiquitously expressed subunits-α5 and -β1. Although subunit-α2 expression was greater on TSC2-null cells than TSC2-positive cells and α2β1 has been implicated in cell migration in other cell types, the level of expression was very low, and therefore we did not pursue this integrin.

Upregulation of specific integrin receptors during cancer progression has been reported in many studies; for example, α1β1, α2β1 and α3β1 are upregulated in hepatocarcinoma (22, 36) and are associated with the acquisition of a migratory capacity of the cells. Our results demonstrate that inhibition of α1 alone or in combination with β1 reduced migration of TSC2-null cells by 25–30%. Similarly integrin subunits-α1, -α2, and -β1 have been implicated in hepatocarcinoma cell invasion (37), in the growth of lung epithelial tumors (26), and lymphangiogenesis (17). Despite our finding that the expression and role of the integrin-α1 subunit in cell migration was increased, inhibition of α1 had no effect on the enhanced attachment and spreading of the cells. The lack of effect of blocking β1 on cell migration was surprising, as inhibition of β1 reduced cell attachment. These findings suggest that the α1β1-integrin may be important for enhanced migration and that the individual subunits may have differential effects on attachment and migration modulated by TSC2 loss.

The interaction between TSC2 and integrins is not fully understood; however, focal adhesion kinase (FAK) may play a role. FAK is a protein tyrosine kinase that mediates various signal transduction pathways initiated by ECM-β-integrin interactions. Gao et al. (7) proposed that, upon activation, FAK phosphorylates TSC2, thus destabilizing and inactivating it, resulting in increased S6K activity. Therefore, ECM-integrin interactions may not only increase FAK but could also increase cellular functions, such as spreading or migration. Therefore, function blocking antibodies against integrin-β1 may decrease FAK activity and thus decrease the enhanced response. However, in the current study, blocking β1 had little effect on the increased spreading and migration of TSC2-null cells. It is possible that this may be because, although blocking β1 would decrease FAK activation, in TSC2-null cells the TSC1/TSC2 complex is already dysfunctional, and thus blocking β1 is not as effective.
Our study has demonstrated that loss of TSC2 enhanced cellular attachment, spreading, and migration as well as expression of the integrin subunit-α1 and that inhibition of integrin-α1β1 reduced the enhanced migration of these cells. Thus inhibiting the integrin-α1β1 may provide a novel target for the treatment of diseases where TSC2 is dysfunctional, such as in LAM.

ACKNOWLEDGMENTS

The authors thank Dr. David Kwiatkowski for the gift of the mouse embryonic fibroblasts, Dr. Cheryl Walker for the gift of the TSC2-null ELT3 cells, Dr. Elena Goncharova for input, and Mr. Dmitry Goncharov, Mrs. Irene Khavin, and Mr Patrick Ng for technical support.

GRANTS

This work was funded by the New Zealand LAM Charitable Trust (L. Moir), the LAM Australasia Research Alliance (LARA) (L. Moir; J. Black), and NIH/NHLBI 5R01HL09829 (V. Krymskaya).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: L.M.M. and V.P.K. conception and design of research; L.M.M. performed experiments; L.M.M., J.L.B., and V.P.K. interpreted results of experiments; L.M.M. prepared figures; L.M.M. drafted manuscript; L.M.M., J.L.B., and V.P.K. edited and revised manuscript; L.M.M., J.L.B., and V.P.K. approved final version of manuscript.

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