Effects of Prolactin on *TSC2*-Null Eker Rat Cells and in Pulmonary Lymphangioleiomyomatosis

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Rationale: Lymphangioleiomyomatosis, a cystic lung disease of women, is characterized by proliferation of smooth muscle-like lymphangioleiomyomatosis cells, which possess mutations in the tuberous sclerosis complex genes, *TSC1/TSC2*. Growth factors involved in lymphangioleiomyomatosis cell proliferation are unknown. Prolactin, an important reproductive hormone in women, is known to promote cell proliferation and survival in other tissues. **Objectives:** To determine the role of prolactin in signaling and proliferation in lymphangioleiomyomatosis.

Methods: Prolactin levels in the sera of patients with lymphangioleiomyomatosis were correlated with clinical status. Components of prolactin signal transduction pathways were assessed in lymphangioleiomyomatosis lesions from human lung explants by real-time reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry. Prolactin effects on proliferation and signaling were quantified in tuberin-deficient and tuberin-expressing rat cells *in vitro*.

Measurements and Main Results: Higher prolactin levels in the sera of patients with lymphangioleiomyomatosis were associated with a faster rate of decline in FEV₁ and an increased history of pneumothorax ($P < 0.01$). Higher levels of prolactin and prolactin receptor mRNA and immunoreactivity were found in lymphangioleiomyomatosis lesions when compared with vascular smooth muscle cells in the same region of tissue. This was accompanied by evidence of activation of signal transducer and activator of transcription-1 (STAT1), STAT3, p44/42, and p38 mitogen-activated protein kinase. *Tsc2*^{-/-} Eker rat embryonic fibroblasts expressed more prolactin receptor than did *Tsc2*^{+/+} cells, and responded to prolactin with increased proliferation and activation of the same signaling pathways seen *in vivo*.

Conclusions: Prolactin may be an important growth factor in the pathogenesis of lymphangioleiomyomatosis.

Keywords: proliferation; pneumothorax; pulmonary function tests; tuberous sclerosis complex

Lymphangioleiomyomatosis (LAM) is an uncommon disease, predominantly found in women of childbearing age and characterized by abnormal proliferation of smooth muscle-like LAM cells, causing cystic destruction of the lung, progressive loss of pulmonary function, and, in some patients, lung transplan-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Lymphangioleiomyomatosis is a cystic lung disease characterized by proliferation of abnormal smooth muscle-like cells (LAM cells) with dysfunctional *TSC2*. It affects primarily women and yet little is known about the effect of childbearing hormones and their relationship to disease progression.

What This Study Adds to the Field

Clinically, higher prolactin levels in the sera of patients with LAM were associated with a faster rate of decline in pulmonary function and an increased incidence of pneumothorax. We also show expression of prolactin and its receptor by LAM cells; activation of intracellular signaling pathways involved in prolactin signaling in LAM lesions; and the stimulation of proliferation and activation of the same signaling pathways by prolactin in *Tsc2*^{-/-} cells.

tation or death (1, 2). LAM occurs sporadically or in association with tuberous sclerosis complex (TSC), an autosomal dominant disorder of variable penetrance associated with hamartomatous lesions (3, 4). LAM cells from patients with sporadic LAM, as well as from patients with LAM with TSC, are associated with mutations in tuberous sclerosis genes, *TSC1* or *TSC2*, that result in activation of the mammalian target of rapamycin (mTOR) signaling pathway, leading to dysregulation of cell proliferation, size, and survival (4–7). The reasons why pulmonary disease in LAM occurs predominantly in women are not well understood, but hormones such as estrogen or testosterone may play a role. Indeed, estrogen has been shown to promote tuberin-null cell survival and metastasis to the lung (8).

Prolactin (PRL) is an important hormone for women of childbearing age, with key roles in proliferation, differentiation, and survival of a wide variety of cells (e.g., lymphocytes, breast cancer cells) (9, 10). Although PRL is produced mainly by the anterior pituitary gland and acts systemically as a classical endocrine factor, it is also produced locally by multiple extrapituitary sites, where it acts in an autocrine/paracrine manner (10, 11). It is also produced by human tumors, including breast and lung (9–12).

Prolactin activates cells through the PRL receptor (PRLr), a type I transmembrane protein, which consists of an extracellular domain, a short transmembrane domain, and a variable intracellular domain (ICD) that mediates signaling (9). Alternative splicing generates PRLr isoforms classified by the length of their ICDs as short, intermediate, or long. The signaling properties of the intermediate and short isoforms differ from those of the long form because of different interactions of the shorter ICDs with scaffolding and signaling proteins (9, 13).

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Major signaling networks downstream of PRL/PRLr include Janus kinases (JAKs) and the signal transducers and activators of transcription (STAT) signaling pathways as well as the Shc-Ras-Raf-mitogen-activated protein kinase (MAPK) pathway (9, 10, 13). These pathways impact crucial cellular processes including proliferation, survival, and differentiation (9, 10, 13).

Because LAM lung disease affects primarily premenopausal women, it is possible that hormonal growth factors, such as PRL, that are found in higher concentrations in women versus men could influence disease onset/progression. Further, there are unknown factors that promote proliferation of LAM cells. We found PRL, PRLr, and activated downstream signaling pathways in LAM lung lesions, and also found greater levels of PRLr in Eker rat *Tsc2*^{-/-} cells, along with higher PRL-dependent downstream signaling and cell proliferation, than in *Tsc2*^{+/+} cells. Higher blood levels of prolactin in patients with LAM correlated with a faster rate of decline in FEV₁ and with an increased history of pneumothorax. These findings suggest that PRL signaling may be important in *Tsc2*^{-/-} cells and contribute to LAM pathogenesis.

METHODS

Complete and detailed methods can be found in the online supplement.

Samples from Patients with LAM

This research was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (protocol 95-H-0186), and informed consent was obtained from all participants. Lung tissue samples were taken from a study group of 30 patients with a diagnosis of LAM confirmed by biopsy. Prolactin measurements were performed by the clinical laboratories of the National Institutes of Health. Only patients who were not deemed at risk for acute deterioration were encouraged to travel to the NIH for study. Because the test for prolactin changed over time, as did the normal ranges with each test, each value was converted to a percentage of the highest normal value for that test; this allowed standardization of results and permitted averaging of prolactin levels from different tests. Two hundred and six patients had values for prolactin and pulmonary function tests. Ninety-four had information about pneumothorax history. Methods of pulmonary function testing and calculation of the yearly rate of decline in lung function were done as previously described (14).

Cell Culture

Eker rat embryonal fibroblast (EEF) *Tsc2*^{-/-} and EEF *Tsc2*^{+/+} cells (15) were grown in mesenchymal stem cell basal medium, supplemented with mesenchymal cell growth supplement, L-glutamine, and GA-1000 (Lonza, Walkersville, MD).

Immunohistochemistry and Immunocytochemistry

The intensity of immunostaining for each molecule tested was graded 0–3 for none, weak, moderate, or strong, respectively. We used as negative control for each species nonspecific IgG controls (i.e., goat, rabbit) as described and shown in Figure E1 in the online supplement.

Laser-Capture Microdissection and RNA Isolation

Tissue sections (thickness, 8 μ m) of LAM lungs were microdissected with the Veritas laser capture microdissection system (Arcturus Engineering, Mountain View, CA). A PicoPure RNA isolation kit (Arcturus) was used to extract RNA according to the manufacturer's instructions.

Real-Time Reverse Transcription Polymerase Chain Reaction Amplification

Ready-to-use primer and probe sets (Assays-on-Demand gene expression: product numbers Hs00168730_m1, Hs00168739_m1, and glyceraldehyde-3-phosphate dehydrogenase [GAPDH]; Applied Biosystems, Foster City, CA) were used for the detection of human PRL and human PRLr (long form) mRNAs.

Preparation of Whole Cell Lysates

Cells were incubated for 24 hours in 6-well dishes in mesenchymal medium to allow attachment, before replacement with serum- and phenol red-free RPMI (Invitrogen, Carlsbad, CA). After incubation for 24 to 48 hours, cells were treated with PRL (ovine-PRL; Sigma-Aldrich, St. Louis, MO) and/or S179D-PRL as indicated. Cells were homogenized, lysates were cleared by centrifugation, and the supernatant was termed "whole cell lysate."

Western Blotting

Equal amounts of protein were separated by electrophoresis in a 4–12% NuPAGE Bis-Tris gel system (Invitrogen) and electrotransferred to nitrocellulose (Invitrogen). Immunoreactivity on blots was detected with a luminescence image analyzer with charge-coupled device camera (LAS-4000; Fujifilm, Tokyo, Japan) and quantified by densitometry, using Fuji Image Gauge software (version 4.0; Fujifilm). The quantity of each phosphorylated protein was expressed as a ratio to that of the same total protein, which was quantified after reaction of the stripped blot with appropriate antibodies. Results are reported relative to that of *Tsc2*^{-/-} cells at zero time = 1.0.

Cell Proliferation

Proliferation of cells was quantified as the amount of 5-bromo-2-deoxyuridine incorporated into DNA (Labeling and Detection Kit III; Roche Diagnostic Corp., Indianapolis, IN). During incubation of cells without or with PRL (2 or 10 μ g/ml) and/or S179D-PRL (0.2 or 1 μ g/ml), medium was replaced every other day, until determination of proliferation on Day 5. Absorbance values for triplicate wells were expressed as an activation index defined as the ratio of absorbance of sample incubated with additions to control sample incubated without additions.

Statistical Analysis

The prolactin measurements were log-transformed because their distribution was skewed to the right. Two-group comparisons of continuous variables were done by Student *t* test. Associations of prolactin as a three-group categorical variable with other continuous variables were done by trend test (Jonckheere-Terpstra test). mRNA and protein analyses and cell growth assays with PRL and/or S179D-PRL antagonist were performed at least seven times. For each data set, arithmetic means and SD were calculated. *P* < 0.05 (*P* < 0.01 in Figures 1 and 3–6) were considered to be statistically significant.

RESULTS

Correlation of Blood Levels of Prolactin and LAM Disease Progression

To determine whether levels of prolactin had an effect on disease progression in patients with LAM, pulmonary function characteristics of patients with LAM were examined in relationship to serum prolactin levels. The range of prolactin spanned 2.0–120.0 μ g/L, with an average value $\pm$ SEM of 22.1 $\pm$ 1.0 μ g/L (206 patients). Over the course of our longitudinal study, the clinical test for prolactin changed, as did the range of normal levels. Levels of prolactin were transformed into a percentage of the highest normal value for the test used at the time, thus standardizing the tests and permitting averaging of levels among tests. The average percent $\pm$ SEM was 92.4 $\pm$ 6.4% (range, 7.4–1091%; 206 patients). Prolactin levels were compared with four pulmonary function parameters known to be affected in LAM: initial FEV₁ (percent predicted), initial diffusing capacity of the lung for carbon monoxide (DL_{CO}) (percent predicted), and rates of decline in FEV₁ and DL_{CO} (percent/yr). Prolactin was not a significant linear predictor of the rates of decline in FEV₁ or in DL_{CO}; however, the effect of prolactin on the rate of decline in FEV₁ may not be linear (a linear relationship test rejected this association in favor of a nonlinear one; *P* = 0.001). Three categories of prolactin levels were examined: less than 60,

TABLE 1. ASSOCIATION OF LEVELS OF PROLACTIN WITH PULMONARY FUNCTION MEASUREMENTS

Prolactin*	Initial D _{LCO} (% predicted)	Initial FEV ₁ (% predicted)	Rate of Decline in D _{LCO} (%/yr)	Rate of Decline in FEV ₁ (%/yr)	n†
<60	79.5 ± 3.1	80.1 ± 3.2	2.6 ± 0.7	1.3 ± 0.4	64
60–100	77.9 ± 2.5	77.4 ± 2.6	4.1 ± 0.4	2.6 ± 0.5	91
>100	77.5 ± 3.8	79.6 ± 3.7	2.9 ± 1.0	3.7 ± 0.8‡	51

Definition of abbreviation: D_{LCO} = diffusing capacity of the lung for carbon monoxide.

* Prolactin values represent the percentage of the highest value of the range of normal values. Levels of prolactin were standardized across various clinical tests by determining the percentage of the highest normal level for each test. Samples with values greater than 100 are samples with higher than normal levels of prolactin.

† n = number of patients.

‡ Trend test for rate of decline in FEV₁ versus prolactin categories: $P = 0.001$.

60–100, and more than 100% of the highest normal range value. When the rate of decline in FEV₁ was compared with the three categories of prolactin, a significant trend was seen, with higher prolactin levels associated with a greater rate of decline in FEV₁ ($P = 0.001$) (Table 1).

Patients with LAM frequently have a history of recurrent pneumothorax (14). Patients with a history of pneumothorax were more likely to have high normal or higher than normal serum prolactin levels ($P = 0.006$) (Table 2).

TABLE 2. ASSOCIATION OF LEVELS OF PROLACTIN WITH HISTORY OF PNEUMOTHORAX

Prolactin*	No Pneumothorax	Pneumothorax	P Value
<60	22 (50.0)†	11 (22.0)	0.006‡
60–100	14 (31.8)	22 (44.0)	
>100	8 (18.2)	17 (34.0)	

* Prolactin values represent the percentage of the highest value of the range of normal values. Levels of prolactin were standardized across various clinical tests by determining the percentage of the highest normal level for each test. Samples with values greater than 100 are samples with higher than normal levels of prolactin.

† Data represent number of patients (percent).

‡ P value determined by a trend test (Jonckheere-Terpstra).

PRLr, PRL, and Downstream Signaling in LAM Lung Lesions

To explore the potential role of PRL signaling pathways in LAM lung lesions, we used immunohistochemistry. Serial sections of nodular LAM lesions (Figure 1A) showed strong reactivity with anti-PRLr antibodies (Figure 1B) and anti-PRL antibodies (Figure 1C). Immunoreactivity of cells within LAM lesions was variable. Strong reactivity with anti-PRLr antibodies was present in cytoplasm of both spindle-shaped and epithelioid LAM cells in the LAM lung nodules (Figure 1B), and weaker reactivity was present in vascular smooth muscle cells (data not shown). We used as negative control for each species nonspecific IgG controls (i.e., goat, rabbit) as described and shown in Figure E1.

Figure 1. Prolactin receptor (PRLr) and prolactin (PRL) in lymphangioleiomyomatosis (LAM) lung lesions. Serial sections of a nodular LAM lung lesion (A, hematoxylin-eosin stain) show strong reactivity with (B) anti-PRLr antibodies and (C) anti-PRL antibodies. The cytoplasm of LAM cells shows strong reactivity with both antibodies (B and C; insets show area of higher magnification [$\times 600$]; arrows indicate reactivity). Staining was repeated on 20 patient samples. Stars show the orientation of serial sections. Original magnification: (A–C) $\times 200$. We used as negative control for each species nonspecific IgG controls (i.e., goat, rabbit) as described and shown in Figure E1. LAM lesions from frozen sections of explanted LAM lungs were isolated by laser-capture microdissection (LCM) for quantification of PRL, PRLr, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs by real-time reverse transcription-polymerase chain reaction (RT-PCR). Ratios of amounts of (D) PRLr or (E) PRL mRNA to that of GAPDH in LAM lesions and smooth muscle in vessel walls from the same slides were compared. $n = 10$ lung explants. Real-time RT-PCR products were separated by electrophoresis to confirm size and amounts. Lanes L1 and L2 and lanes V1 and V2 are from LAM lesions and vessel walls, respectively, of two patients. $**P < 0.01$ for differences between amounts of prolactin and prolactin receptor mRNA from LAM lesions and vessel walls.

Figure 2. Phosphorylated Janus kinase-2 (JAK2), signal transducer and activator of transcription-3 (STAT3), and p44/42 in lymphangioleiomyomatosis (LAM) lung lesions. Serial sections (A and B) show that the cytoplasm of spindle-shaped LAM cells has strong reactivity with (A) anti-phospho-JAK2 antibodies and (B) anti-phospho-STAT3 antibodies. Stars show the orientation of serial sections. Original magnification: $\times 400$. Reactivity with anti-phospho-STAT3 antibodies is also seen in the nuclei of LAM cells (B, inset, higher magnification [$\times 600$], arrowhead). (C) LAM lesions also demonstrated strong reactivity with anti-phospho-p44/42 antibodies. Star in (C) shows LAM nodule. Original magnification: $\times 200$.

expressed locally in LAM lung lesions, and thus may have an autocrine effect on LAM cells.

In serial sections, anti-phospho-JAK2 antibodies (Figure 2A) and anti-phospho-STAT3 (Ser-727) antibodies (Figure 2B) reacted with cytoplasmic proteins of spindle-shaped LAM cells. Nuclear reactivity with anti-phospho-STAT3 (Ser-727) antibodies (Figure 2B) was also seen in cells of LAM lung lesions. LAM lung lesions also reacted with anti-phospho-p44/42 antibodies (Figure 2C).

Immunoreactivity was graded 0–3 for none, weak, moderate, and strong, respectively. Most LAM lesions contained weak to moderate reactivity with antibodies against PRL (staining intensity, mean $\pm$ SEM [n]: PRL, 1.4 ± 0.2 [20]; PRLr, 1.8 ± 0.1 [20]) and phosphorylated downstream signaling molecules (phospho-JAK2, 2.4 ± 0.2 [9]; phospho-STAT3, 0.8 ± 0.2 [8]; phospho-p44/42, 1.3 ± 0.3 [10]). These results suggested that, as in breast and prostate cancer (9, 10, 12, 13, 16), PRL may act in an autocrine/paracrine manner in LAM lung lesions.

PRLr Protein in *Tsc2*^{−/−} Cells and *Tsc2*^{+/+} Cells

To evaluate PRL signaling in cells that lack tuberlin function, we used well-characterized Eker rat embryonal fibroblasts (EEFs), both *Tsc2*^{−/−} cells, which lack tuberlin, and *Tsc2*^{+/+} cells that express tuberlin. By Western blotting, amounts of PRLr long form, but not short form, in *Tsc2*^{−/−} cells were greater than in *Tsc2*^{+/+} cells (Figures 3A–3C). Similarly, ratios of PRLr long to short levels were higher in *Tsc2*^{−/−} than *Tsc2*^{+/+} cells (Figure 3).

Effect of PRL on *Tsc2*^{−/−} and *Tsc2*^{+/+} Cells

To determine PRL effects on signaling events, *Tsc2*^{−/−} cells were incubated with ovine PRL and protein was analyzed by Western blotting. Because signaling networks downstream of PRL/PRLr include JAK/STAT and MAPK pathways, we looked for phosphorylation of STAT1, STAT3, p38, and p44/42. In *Tsc2*^{−/−} cells, maximal phosphorylation of STAT1, STAT3, p38, and p44/42 was observed 0.5 hour after incubation with PRL (Figure 4A). We observed significant activation of STAT1 (Figure 4B), STAT3 (Figure 4C), p44/42 (Figure 4D), and p38 (Figure 4E) in response to prolactin by *Tsc2*^{−/−} cells. In contrast, *Tsc2*^{+/+} cells were insensitive to this growth factor (Figures 4A–4E). Moreover, for the *Tsc2*^{−/−} cells, STAT1, STAT3, p38, and p44/42 were activated by PRL in a dose-dependent manner (Figures 4F–4J). These results are consistent with our hypothesis that PRL/PRLr signaling may have important effects on these regulatory pathways in tuberlin-deficient cells.

To explore a consequence of PRL stimulation, we measured cell proliferation, as assessed by bromodeoxyuridine incorporation. Consistent with activation of STAT1, STAT3, p44/42, and p38 in these cells, PRL induced greater proliferation of *Tsc2*^{−/−} cells than of *Tsc2*^{+/+} cells (Figure 5A).

To quantify PRLr and PRL mRNA expression in LAM lung lesions we used laser-capture microdissection (LCM), followed by real-time reverse transcription-polymerase chain reaction (RT-PCR). Consistent with the results of immunohistochemistry, ratios of PRLr and PRL mRNAs normalized to GAPDH mRNA were significantly higher in LAM lesions than in vessel walls of the same frozen sections (Figures 1D and 1E). Thus, using a quantitative measure, we conclude that PRL is

Figure 3. Prolactin receptor (PRLr) protein in *Tsc2*^{-/-} cells and *Tsc2*^{+/+} cells. (A) Western blot analysis of PRLr in Eker rat embryonal fibroblast (EEF) *Tsc2*^{-/-} cells and *Tsc2*^{+/+} cells. Proteins in lysates of serum-deprived *Tsc2*^{-/-} and *Tsc2*^{+/+} cells were analyzed with antibodies against PRLr, tuberlin, and α-tubulin (gel-loading control). Immunoreactive 84-kD PRLr long was present in all samples, but amounts were greater in *Tsc2*^{-/-} lysates. In seven experiments like that in (A), amounts of proteins were quantified by densitometry and expressed relative to that of α-tubulin in the same samples: (B) PRLr long/α-tubulin and (C) PRLr short/α-tubulin. (D) Ratios of PRLr long to PRLr short in *Tsc2*^{-/-} and *Tsc2*^{+/+} cells. **P* < 0.05 for differences between *Tsc2*^{-/-} and *Tsc2*^{+/+} cells. N.S. = not significant.

Inhibition of PRL Stimulation of *Tsc2*^{-/-} and *Tsc2*^{+/+} Cell Proliferation by the PRLr Antagonist S179D-PRL

To confirm that the PRL effects were mediated by interaction with PRLr, we investigated the effect of a PRLr antagonist on PRL stimulation. S179D-PRL binds to both long and short receptors (17), inhibits some signaling by PRL through the long receptor isoform (18, 19), and alters splicing of PRLr, favoring production of the PRLr short isoform (18–21). This resulted in reduced cell proliferation in a number of *in vivo* and *in vitro* systems (18, 19). In *Tsc2*^{-/-} cells, PRL (10 μg/ml)-dependent cell proliferation was inhibited by S179D-PRL (1 μg/ml) (Figure 5B).

Levels of the PRLr short isoform in *Tsc2*^{-/-} cells were increased by S179D-PRL, as seen on Western blots (Figure 6A). In *Tsc2*^{+/+} cells, no effect of PRL or S179D-PRL was seen. Differences between *Tsc2*^{-/-} and *Tsc2*^{+/+} cells were evident in ratios of PRLr short to long isoform (Figure 6B), with amounts of PRLr long greater in *Tsc2*^{-/-} cells than in *Tsc2*^{+/+} cells (Figure 6C), but not altered significantly by treatment with S179D-PRL.

DISCUSSION

In patients with LAM, the rate of decline in FEV₁ is associated with levels of prolactin, with higher levels of prolactin resulting in greater rates of decline (Table 1). Previously, we determined that the decline in FEV₁ was greater in patients with a predominantly solid pattern of LAM lesions, or more nodules of LAM cells, than in those patients without this pattern (22). Prolactin may stimulate the proliferation of the LAM cells, leading to faster rates of decline in FEV₁. Indeed, a large prospective study has shown increased risk of development of breast cancer with higher PRL levels (23, 24). Concordant with this proposed effect of prolactin on cell growth, we show that prolactin stimulated

proliferation of *Tsc2*^{-/-} cells (Figure 5A). Proliferation and/or stimulation of LAM cells may also lead to a greater production of matrix metalloproteinases, perhaps contributing to the greater chance of pneumothorax with higher levels of prolactin. Although prolactin production can be stimulated by stress (25, 26), patients examined here were not acutely ill, and therefore associations between the prolactin level and the rate of decline in FEV₁ were probably not due to nonspecific effects caused by acute stress. The effects of prolactin appear to be independent of a stress response; however, the immunological effects of prolactin on LAM patients remain to be studied (25, 26).

Here, we report the presence of PRL, PRLr, and activated signaling molecules downstream of PRLr in LAM lung lesions, and effects of PRL on rat *Tsc2*^{-/-} cells and *Tsc2*^{+/+} cells. Levels of PRL and PRLr were higher, as were those of activated downstream signaling molecules, in LAM lung lesions compared with those in adjacent vascular smooth muscle. We also found greater responses to PRL in *Tsc2*^{-/-} cells than in *Tsc2*^{+/+} cells. Also, a PRLr antagonist inhibited PRL-dependent proliferation of *Tsc2*^{-/-} cells and appeared to change PRLr splicing, as manifested by an increased ratio of short to long receptor isoforms. These data suggest that altered PRL signaling in tuberlin-deficient cells may have a role in the pathogenesis of LAM.

Although the pituitary gland is a primary site of its production, prolactin has also been detected in many extrapituitary sites, for example, mammary epithelium, endometrial stromal cells, umbilical vein endothelial cells, lymphocytes, adipocytes (10, 11, 27), and breast and prostate cancer cells (12, 16), where it may act as an autocrine or paracrine growth factor (9–11). PRL and PRLr mRNAs have been reported to be significantly elevated in cancerous versus adjacent noninvolved tissue of the same patient in breast and colorectal cancer (28, 29). Because LAM cells have phenotypic characteristics similar to smooth muscle cells, we used vascular smooth muscle for comparisons.

Figure 4. Effect of prolactin (PRL) on *Tsc2*^{-/-} cells: time-dependent and concentration-dependent activation of signal transducer and activator of transcription-1 (STAT1), STAT3, p38, and p44/42. **Panel I:** Serum-deprived Eker rat embryonal fibroblast (EEF) *Tsc2*^{-/-} and *Tsc2*^{+/+} cells were incubated with PRL (10 μg/ml) for 0 minutes, 15 minutes, 30 minutes, 60 minutes, 120 minutes, or 24 hours before preparation of cell lysates and Western blotting of protein samples (25 μg) for phosphorylated and total STAT1, STAT3, p38, and p44/42. (A) Pairs of blots show reactions with antibodies against phosphorylated STAT1, STAT3, p38, and p44/42 (top rows); the stripped membranes were reprobbed with antibodies against total STAT1, STAT3, p38, and p44/42 (bottom rows). (B–E) Data from experiments like that in (A) were quantified by densitometry and ratios of the same protein (phosphorylated to total) in each sample were expressed relative to that in *Tsc2*^{-/-} cells at zero time (= 1.0). Results are reported as means ± SD of values from seven experiments. **Panel II:** Serum-deprived *Tsc2*^{-/-} and *Tsc2*^{+/+} cells were incubated with PRL (0, 0.06, 0.3, 1.5, 5, or 10 μg/ml) for 30 minutes, before preparation of cell lysates and Western blotting of protein samples (25 μg) from each. (F) Pair of blots show reactions with antibodies against phosphorylated STAT1, STAT3, p38, or p44/42 above the stripped membrane reprobbed with antibodies against total STAT1, STAT3, p38, or p44/42. (G–J) Data from experiments like that shown in (F) were quantified by densitometry. Ratios of each phosphorylated to total protein in the same sample were expressed relative to that in untreated *Tsc2*^{-/-} cells (= 1.0) in the same experiment. Data are reported as means ± SD of values from seven experiments. **P* < 0.05 for differences between indicated means.

We found amounts of PRL mRNA in LAM lesions greater than those in vessel walls in the vicinity of the lesions. Although we did not detect reactivity with anti-PRL antibodies in vascular

smooth muscle, we were able to detect PRL mRNA in microdissected samples from vascular walls. In the lung, lymphocytes and mononuclear cells would also be included as sources of

Figure 5. Effects of prolactin (PRL) and S179D-PRL (S179D) on proliferation of *Tsc2*^{-/-} and *Tsc2*^{+/+} cells, as quantified by bromodeoxyuridine (BrdU) incorporation. Serum-deprived (18 h) Eker rat embryonal fibroblast *Tsc2*^{-/-} and *Tsc2*^{+/+} cells were seeded in 96-well plates (3×10^3 cells per well) in phenol red-free RPMI-0.1% charcoal-stripped fetal bovine serum medium with PRL (2 or 10 µg/ml) and/or S179D-PRL (0.2 or 1 µg/ml), incubated for 96 hours before addition of BrdU, and analyzed 18 hours later. Data are expressed relative to that of unstimulated cells for each group (= 1.0) (controls). Means $\pm$ SD of values from (A) nine experiments or (B and C) seven experiments are shown. * $P < 0.05$, ** $P < 0.01$ for the indicated differences.

local PRL (27, 30). It is possible, therefore, that those cells could be responsible for the PRL mRNA seen in vascular walls. However, because we did not find any notable accumulation of lymphocytes and mononuclear cells by histological analysis of LAM lesions, this result suggests that LAM cells produce PRL mRNA. In our studies, we used various concentrations of

Figure 6. Effect of prolactin (PRL) and S179D-prolactin (S179D) on amounts of long and short forms of prolactin receptor (PRLr) in *Tsc2*^{-/-} and *Tsc2*^{+/+} cells. (A) Serum-deprived (12 h) Eker rat embryonal fibroblast *Tsc2*^{-/-} and *Tsc2*^{+/+} cells were incubated for 36 hours with PRL (10 µg/ml) and/or S179D-PRL (0.02, 0.2, or 1 µg/ml), before analysis of PRLr, tuberin, and α -tubulin by Western blotting. Data from seven experiments, like that shown in (A), were quantified by densitometry for comparison of amounts of PRLr (B, ratio of short to long form; C, ratio of long form to α -tubulin) after normalization to the α -tubulin level. Results are expressed relative to that in untreated cells (= 1.0). * $P < 0.05$ for the indicated differences. N.S. = not significant.

prolactin, but we found that higher concentrations of prolactin were required to stimulate cell proliferation than were found in the sera of patients with LAM. The discrepancy between the circulating levels of prolactin and the concentrations of prolactin used in the proliferation assays may be reconciled if the local concentration of prolactin is high. As the LAM nodule is thought to produce prolactin, the microenvironment of the nodule may play a role in LAM cell proliferation. Similar effects of microenvironment and extracellular matrix have been noted (31).

In addition to reports of higher levels of PRLr mRNA and protein in breast tumor tissue or breast cancer cell lines than in

surrounding normal tissue or a normal breast cell line (32, 33), a difference in the ratio of PRLr long to short has also been described (34). Using real-time PCR, it was found that breast tumor tissue and breast cancer lines have higher ratios of PRLr long to short than does normal breast tissue (34), suggesting that either signaling through the long form or lack of the short form (or both) promotes cancer. Consistent with observations in breast cancer, we detected more PRLr long mRNA in LAM lung lesions than in smooth muscle of vessel walls near the lesions (Figure 1). In addition, amounts of long PRLr were elevated in *Tsc2*^{-/-} cells, resulting in an increased ratio of long to short receptors and a proliferative response to added PRL. Collectively, our findings suggest that PRL, acting through the long receptor, may function as an autocrine/paracrine growth factor in LAM lung lesions. In addition, correlations between measures of disease progression and levels of circulating PRL suggest either that there are contributions by circulating prolactin or that LAM lesions produce enough PRL to elevate circulating PRL.

In addition to inhibiting growth-promoting signals at the long PRLr (35), S179D-PRL also changes PRLr splicing, increasing the short form of PRLr and affecting the ratio of PRLr short to long (20, 21). Consistent with results in other systems, our data show that S179D-PRL changed the amount of the PRLr in rat *Tsc2*^{-/-} cells, increasing the level of PRLr short and decreasing PRL-stimulated proliferation (Figures 5 and 6).

The main signaling networks from PRL/PRLr include the JAK/STAT and MAPK pathways. In human breast cancer cells, PRL secretion constitutively increased cell proliferation via activated JAK/STAT and MAPK pathways (9, 10, 13, 36). Our findings of strong immunoreactivity for PRL/PRLr and the presence of activated downstream signaling molecules in LAM lung lesions, for example, phospho-JAK2, phospho-STAT3, and phospho-p44/42 MAPK (Figures 1 and 2), are consistent with the hypothesis that PRL signaling has an important role in the pathogenesis of LAM, although the possibility that these pathways are also activated through other receptors and their ligands in LAM lesions cannot be excluded.

To investigate the role of PRL signaling in tuberin-deficient cells, we used Eker rat *Tsc2*^{-/-} cells as a cell model for LAM (37) and showed that in these cells, activation of JAK/STAT and MAPK pathways is promoted by PRL. Thus in these cells, we observed activation of the same pathways as in the LAM lesions. We demonstrated that PRL strongly activated STAT1, STAT3, p44/42, and p38 MAPK pathways in *Tsc2*^{-/-} cells, but not in *Tsc2*^{+/+} cells (Figure 4). However, in both types of cell, we did not detect strong activation of STAT5 (data not shown), which is a major mediator of both differentiation and proliferation of other cells in response to PRL (9, 10, 35, 38). Collectively, these observations suggest a model in which loss of *TSC2* function is associated with enhancement of some PRL signal pathways to promote cell growth.

Tsc1^{-/-} and *Tsc2*^{-/-} mouse embryo fibroblasts and *Tsc1*^{+/-} and *Tsc2*^{+/-} renal and liver tumor cells showed increased STAT1 (Ser-727) and STAT3 (Tyr-705) phosphorylation (39). Further, LAM lung and renal AML lesions exhibit increased STAT1 (Ser-727) and STAT3 (Tyr-705) phosphorylation (40). These findings are similar to our data showing enhanced PRL effects in *Tsc2*^{-/-} cells, as well as greater PRL-induced phosphorylation of Ser-727 in STAT1 and STAT3 than in *Tsc2*^{+/+} cells, and reactivity with antibodies against phospho-STAT3 (Ser-727) in LAM lesions. Although many growth factors and cytokines can activate STAT1 and STAT3, our data on PRL and PRLr in LAM lesions and preferential activation of STAT pathways in *Tsc2*^{-/-} cells by PRL lead us to hypothesize that

LAM lung lesions may be subject to a similar perturbation of STAT pathway regulation involving PRL.

Relationships between *TSC2* function and MAPK pathways have also been found. First, tuberin is phosphorylated by extracellular signal-regulated kinase (ERK)/MAP kinase. Second, ERK phosphorylation was higher in *Tsc2*^{-/-} cells treated with estrogen, leading to cell proliferation (41). Third, overexpression of tuberin in *Tsc2*^{-/-} cells led to a decrease in estrogen-dependent ERK phosphorylation, resulting in reduced cell proliferation (42). Fourth, *Tsc2*^{-/-} cells, but not *Tsc2*^{+/+} cells, have unique cellular growth pathways involving mitochondrial oxidant-dependent p42/44 MAPK activation in response to platelet-derived growth factor (41). Thus, tuberin both regulates and is regulated by ERK/MAPK. Our finding of preferential activation of MAPK pathways in *Tsc2*^{-/-} cells by PRL is consistent with these findings (Figure 4). Overall, it is reasonable to speculate that loss of *TSC2* function results in dysregulated cell growth through activated STAT and MAPK pathways that could act synergistically with hormonal and growth factor factors, such as PRL, in LAM cells in women.

In conclusion, the high expression of PRL and PRLr with activated PRL signaling molecules in LAM lesions and the enhancement by PRL of *Tsc2*^{-/-} cell growth in culture, with robust activation of PRLr-dependent signaling pathways, suggest that PRL signaling may contribute to the pathogenesis of LAM. PRL may promote tumor growth in an autocrine/paracrine manner in LAM lesions, similar to its actions in breast cancer, leading to a greater rate of decline in FEV₁ and an increased incidence of pneumothorax. These new findings might prove the basis for a therapeutic strategy in LAM, with a receptor antagonist inhibiting proliferation of PRL-activated LAM cells.

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