MOLECULAR PATHOGENESIS OF GENETIC AND INHERITED DISEASES

Inactivation of *Tsc2* in Mesoderm-Derived Cells Causes Polycystic Kidney Lesions and Impairs Lung Alveolarization

Siying Ren,^{*,†} Yongfeng Luo,[†] Hui Chen,[†] David Warburton,^{†‡} Hilaire C. Lam,[§] Larry L. Wang,[†] Ping Chen,^{*} Elizabeth P. Henske,[§] and Wei Shi^{†‡}

From the Department of Respiratory Medicine,^{*} The Second Xiangya Hospital, Central-South University, Changsha, People's Republic of China; the Developmental Biology and Regenerative Medicine Program,[†] The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, California; the Department of Surgery,[‡] Keck School of Medicine, University of Southern California, Los Angeles, California; and the Division of Pulmonary and Critical Care Medicine,[§] Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts

Accepted for publication
August 29, 2016.

Address correspondence to Wei Shi, M.D., Ph.D., Children's Hospital Los Angeles, 4650 Sunset Blvd, MS 35, Los Angeles, CA 90027; or Elizabeth P. Henske, M.D., Thorn 826, Brigham and Women's Hospital, 45 Francis Street, Boston, MA 02115. E-mail: wshi@chla.usc.edu or ehenske@bwh.harvard.edu.

The tuberous sclerosis complex (TSC) proteins are critical negative regulators of the mammalian/mechanistic target of rapamycin complex 1 pathway. Germline mutations of *TSC1* or *TSC2* cause TSC, affecting multiple organs, including the kidney and lung, and causing substantial morbidity and mortality. The mechanisms of organ-specific disease in TSC remain incompletely understood, and the impact of TSC inactivation on mesenchymal lineage cells has not been specifically studied. We deleted *Tsc2* specifically in mesoderm-derived mesenchymal cells of multiple organs in mice using the *Dermo1-Cre* driver. The *Dermo1-Cre*-driven *Tsc2* conditional knockout mice had body growth retardation and died approximately 3 weeks after birth. Significant phenotypes were observed in the postnatal kidney and lung. Inactivation of *Tsc2* in kidney mesenchyme caused polycystic lesions starting from the second week of age, with increased cell proliferation, tubular epithelial hyperplasia, and epithelial-mesenchymal transition. In contrast, *Tsc2* deletion in lung mesenchyme led to decreased cell proliferation, reduced postnatal alveolarization, and decreased differentiation with reduced numbers of alveolar myofibroblast and type II alveolar epithelial cells. Two major findings thus result from this model: inactivation of *Tsc2* in mesoderm-derived cells causes increased cell proliferation in the kidneys but reduced proliferation in the lungs, and inactivation of *Tsc2* in mesoderm-derived cells causes epithelial-lined renal cysts. Therefore, *Tsc2*-mTOR signaling in mesenchyme is essential for the maintenance of renal structure and for lung alveolarization. (*Am J Pathol* 2016, 186: 3261–3272; <http://dx.doi.org/10.1016/j.ajpath.2016.08.013>)

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder resulting from germline loss-of-function mutations in the *TSC1* or *TSC2* gene.^{1,2} The *TSC1/TSC2* protein complex has GTPase-activating protein activity toward Rheb, an upstream regulator of mTOR, and thus negatively regulates mammalian/mechanistic target of rapamycin complex 1 (mTORC1) signaling activity by converting active Rheb-GTP to inactive Rheb-GDP. Therefore, abrogation of *TSC1* or *TSC2* causes dysregulation of mTORC1-dependent pathways, including protein translation, pyrimidine and purine biosynthesis, and autophagy. In addition to neurological disease, which includes

seizures and autism, TSC patients have clinical manifestations in multiple organs, including the kidney, heart, lung, and skin. In the kidney, a variety of lesions, such as angiomyolipoma, isolated or multiple renal cysts, and renal cell carcinoma, can occur in TSC patients. In the lung, TSC

Supported by NIH grants HL068597 (W.S.), HL118760 (E.P.H.), and DK096556 (E.P.H.); a Tuberous Sclerosis Alliance Research grant (W.S.); the Hunan Province Health Department Research Fund B2014-020 (S.R.); the Hunan Province Natural Science Foundation of China 14JJ7012 (S.R.); LAM Foundation Postdoctoral Fellowship (H.C.L.); and the Engles Program in TSC and LAM Research (E.P.H.).

Disclosures: None declared.

patients can develop multifocal micronodular pneumocyte hyperplasia and lymphangioleiomyomatosis (LAM).^{3,4} LAM is a progressive disease affecting almost exclusively women and resulting in cystic lung degeneration. Up to 80% of women with TSC have multiple lung cysts by the age of 40 years, although only a minority will develop symptomatic LAM. Men with TSC can also develop cystic lung disease, although it is almost always asymptomatic. LAM also occurs in a sporadic form in women who do not have TSC. Sporadic LAM is associated with somatic inactivation of the *TSC1* or *TSC2* gene.^{5,6} The pathogenic mechanisms underlying the development of the clinical manifestations of TSC, in particular the pathogenesis of LAM, are not completely understood. One major challenge is the limitation of existing TSC disease models because conventional homozygous *Tsc1* or *Tsc2* knockout mice are embryonic lethal, and heterozygous *Tsc1* or *Tsc2* knockout mice develop epithelial renal lesions and tumors as their primary phenotypes.^{7–9} This is a striking contrast to human TSC, in which mesenchymal lesions of the kidney (angiomyolipomas) and lung (LAM) are major causes of morbidity and mortality. It is still unclear how the TSC protein complex functions in different cell compartments, such as epithelial cells versus mesenchymal cells, during organ development and during post-developmental tissue homeostasis. Although genetic deletions of *Tsc1* or *Tsc2* in distinct cell lineages, such as neurons, neural crest-derived cells, endothelia, cardiomyocytes, and differentiated smooth muscle cells, have been reported in mice,^{10–14} the consequences of *Tsc1* or *Tsc2* inactivation in pan-mesenchymal cells has remained unknown.

We have investigated the specific function of *Tsc2* in mesoderm-derived cells from the beginning of development *in vivo*. We achieved this by crossing a mesoderm-specific Cre-expressing mouse line *Dermo1-Cre* with floxed-*Tsc2* mice to generate mesenchyme-specific *Tsc2* conditional knockout mice.^{15,16} Although these *Tsc2* conditional knockout mice were alive postnatally, postnatal growth retardation, polycystic kidney pathology, and defective lung alveolarization were detected during postnatal development. These mice died approximately 3 weeks after birth, consistent with an indispensable role of *Tsc2* in mesenchymal cells in regulating both organ development and tissue homeostasis.

Materials and Methods

Mouse Strains and Breeding

Mesenchyme-specific *Dermo1-Cre* mice were gifts from Dr. David Ornitz (Washington University, St. Louis, MO).¹⁵ Floxed-*Tsc2* (*Tsc2*^{fl/fl}) mice were gifts from Dr. Michael Gambello's laboratory (Emory University, Atlanta, GA).¹⁶ *mT-mG* double fluorescence reporter mice and *Gt(ROSA)26Sor^{tm1Sor}* LacZ reporter mice were obtained from Jackson Laboratory (Bar Harbor, ME).^{17,18} All mice were bred in C57BL/6 strain background, and housed in pathogen-free

conditions at the animal facility of Children's Hospital Los Angeles. The procedures for mouse breeding and tissue harvest were approved by the Institutional Animal Care and Use Committee at the Saban Research Institute of Children's Hospital Los Angeles. Mouse genotypes were determined by genomic DNA PCR using tail biopsy samples. The genotyping PCR primers were described in previous publications.^{16,19}

Histology and Morphometric Analysis

Fresh tissues at different ages were harvested from euthanized mice, and examined under dissecting microscope. Samples were then either fixed in 4% buffered paraformaldehyde or frozen in liquid nitrogen. The fixed tissues were dehydrated and embedded in paraffin, and cut into sections (5 μ m thick). The tissue sections were stained with hematoxylin and eosin or periodic acid-Schiff (Sigma Aldrich, St. Louis, MO). Lung elastin was stained using Hart's resorcin-fuchsin method, as described in our previous publication.¹⁹ For lung morphometric analysis, five sections of each tissue were randomly chosen at approximately 200- μ m intervals and stained with hematoxylin and eosin. The mean linear intercept was used to measure average size of alveoli, as described previously.¹⁹ Results were analyzed with unpaired *t*-tests to compare the differences between mean values, and considered significant if *P* < 0.05. To avoid the sex differences in organ maturation, this quantitative comparison was also performed among fetuses with different sex at each time point.

Immunostaining and Detection

Tissue sections were deparaffinized and hydrated, and antigen recovery was performed by boiling tissue slides in Tris-EDTA buffer (pH 9.0) for 30 minutes. The tissue sections were blocked in 5% donkey normal serum and 0.5% Triton X-100 for 1 hour, and then incubated with the following primary antibodies overnight at 4°C: mouse anti-green fluorescent protein (GTX628528; GeneTex, Irvine, CA); goat anti-platelet endothelial cell adhesion molecule 1 (sc-1506), goat anti-CC10 (sc-9772), and goat anti-Sp-C (sc-7706; Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-red fluorescent protein for Tomato protein (600-401-379; Rockland, Limerick, PA); rat anti-Cdh1 (13-1900; ThermoFisher, Waltham, MA), mouse anti- β -tubulin IV (MU178-UC; BioGenex, Fremont, CA); fluorescein-*Lotus tetragonolobus* lectin and rhodamine-*Dolichos biflorus* agglutinin (FL-1321 and RL-1032; Vector Laboratories, Burlingame, CA); mouse anti-cytokeratin and mouse anti- α -smooth muscle actin (C2562 and A2547; Sigma Aldrich); rabbit anti-phospho-S6 (Ser235/236) and rabbit anti-vimentin (4858 and 5741; Cell Signaling Technology, Danvers, MA); and hamster anti-T1 α (DSHB at the University of Iowa, Iowa City). Alexa Fluor 488-, Alexa Fluor 594-, or Alexa Fluor 647-conjugated donkey secondary antibodies (ThermoFisher) were used, and cell

nuclei were counterstained by DAPI in the mounting medium (Vector Laboratories). Fluorescence images were taken using the Zeiss LSM710 confocal microscope (Carl Zeiss, Oberkochen, Germany) at the Imaging Core Facility of Children's Hospital Los Angeles. Active caspase 3 staining was performed by immunohistochemistry using rabbit anti-cleaved caspase 3 antibody (9661; Cell Signaling Technology) and second anti-rabbit antibody—horseradish peroxidase conjugate, followed by color development with 3,3'-diaminobenzidine and hematoxylin counterstaining.

Western Blot

Detection of proteins in tissue lysate by Western blot has been previously described with the following minor modification.²⁰ Equal amounts (50 µg) of total tissue lysate proteins were separated in mini tris-glycine extended precast gels (4% to 15% gradient; Bio-Rad, Hercules, CA) and transferred into polyvinylidene difluoride membrane using Bio-Rad's Trans-Blot Turbo Transfer System. Proteins of interest were detected using the following specific antibodies: rabbit anti-Tsc2 (GTX61245; GeneTex); rabbit anti-phospho-S6 (Ser235/236) and mouse anti-S6 (4858 and 2317; Cell Signaling Technology); and rabbit anti-β-actin (G046; Applied Biological Materials, Richmond, BC, Canada).

Cell Proliferation

To detect proliferating cells *in vivo*, cell nuclear 5'-ethynyl-2'-deoxyuridine (EdU) labeling was used.²¹ Briefly, mice were i.p. injected with EdU (5 mg/kg body weight; ThermoFisher) 3 hours before harvesting tissue samples. EdU is a thymidine analogue that is incorporated into newly synthesized chromosome DNA. Incorporated EdU was detected using Alexa Fluor azide (Life Technologies), and cell nuclei were counterstained with DAPI. The image analysis was performed in four random fields per slide from a total of four slides per mouse by an experienced observer (Y.L.) blind to the mouse genotype. The ratios of EdU-labeled nuclei/total DAPI-stained nuclei were used to evaluate cell proliferation, and the experiments were repeated in more than three mice within each genotype group.

Serum Cystatin C Measurement

Blood was collected from five *Tsc2* conditional knockout (CKO) and wild-type (WT) littermate control pairs around postnatal day 20 (P20). The sera were diluted 200-fold, and used for cystatin C measurement using a commercial enzyme-linked immunosorbent assay kit (ThermoFisher). Paired sample *t*-test was used for the analysis.

Statistical Analysis

All experiments were repeated at least three times. The quantitative data were presented as means ± SD. Statistical

analyses were performed using *t*-tests, with $P \leq 0.05$ considered significant.

Results

Conditional Deletion of *Tsc2* in Mesoderm-Derived Cells Results in Postnatal Growth Retardation and Lethality

It has been reported that *Dermo1* promoter-driven *Cre* expression is specific for certain lineages of mesoderm-derived cells in a variety of organs in the *Dermo1-Cre* knockin fetal mice,¹⁵ and we verified this *Cre* expression pattern in embryonic day 14.5 *Dermo1-Cre* fetuses using *Gt(ROSA)26Sor^{tm1Sor}* LacZ reporter mice (Figure 1A). We crossed *Dermo1-Cre* with floxed-*Tsc2* mice (*Tsc2^{fx/fx}*) to specifically delete *Tsc2* gene in *Dermo1*-expressing lineages of mesenchymal cells within multiple organs/systems during development. The mice were born with the expected mendelian ratio of genotypes and sexes. All control pups had normal development and weight gain with normal survival through 12 months of age. *Dermo1-Cre/Tsc2^{fx/fx}* conditional knockout (abbreviated as *Tsc2* CKO) mice were born with notably smaller body size and lower body weight than their littermate controls. These differences became more obvious during postnatal growth (Figure 1, B and C), suggesting significant postnatal growth retardation in *Tsc2* CKO mice. Heterozygous *Tsc2* CKO (*Dermo1-Cre/Tsc2^{fx/+}*) mice had body weight and size comparable to their WT littermates, and did not develop noticeable phenotypic abnormalities up to 5 months of age (data not shown).

The homozygous *Tsc2* CKO mice died approximately 3 weeks after birth, with median survival of P20 (Figure 1D). No sex difference was observed for *Tsc2* CKO mouse survival. Unlike the phenotypic changes seen in liver, cardiac, and central neuronal systems of germline *Tsc2* mutant animals,^{8,22,23} there were no histologically obvious abnormalities in brain, liver, or heart in the *Tsc2* CKO mice at P14 (Figure 2). In contrast, the kidney and lung were clearly abnormal at this age (see Results below for detail). The potential causes for the lethality in *Tsc2* CKO mice are further analyzed and discussed below.

Mesodermal *Tsc2* Abrogation Results in Severe Polycystic Kidney Lesions

Dermo1-Cre—driven gene deletion, shown by green fluorescent protein expression in a mT-mG reporter line,¹⁷ occurred in renal mesenchymal cells of both the cortical and medullary regions, but not in tubular epithelial cells or endothelial cells, shown by *Cdh1⁺* (E-cadherin⁺) and platelet endothelial cell adhesion molecule 1⁺ staining, respectively (Figure 3, A and B). Reduction of *Tsc2* protein and activation of the mTORC1 pathway (increased phosphorylation of ribosomal protein S6) were verified by Western blotting of the entire *Tsc2* CKO kidney tissue lysate (Figure 3C). Examination of the moribund *Tsc2* CKO mice sacrificed at approximately 3 weeks after birth revealed obvious changes in kidney size and appearance, which

looked pale, polycystic, and enlarged by gross view (Figure 3D). We then analyzed the dynamic changes of kidney morphology in the *Tsc2* CKO mice. Interestingly, no significant alterations of kidney structure and size were observed in neonatal *Tsc2* CKO mice at P1 (Figure 4, A and C). Microscopic cystic pathology was observed in the renal cortex from the second week after birth (P14) (Figure 4). The cystic lesions rapidly progressed and expanded into the medulla, destroying the normal renal cortex-medulla structural relationships at 3 weeks of age in all *Tsc2* CKO mice (Figure 4). Glycogen accumulation was detected in the cysts by periodic acid-Schiff staining. In addition, multiple foci of tubular epithelial hyperplasia and adenomatous changes adjacent to the cysts were seen in *Tsc2* CKO kidney after 2 weeks of age (Figure 4, B and C). Consistently, overall cell proliferation in *Tsc2* CKO kidneys was doubled compared to that in WT littermates, shown by nucleotide analogue EdU labeling (9.0 ± 2.4 in *Tsc2* CKO versus 4.1 ± 1.1 in WT) (Figure 5, A and B) at P7 before obvious morphological changes occurred. The cystic changes were further analyzed by staining the tissue with *L. tetragonolobus* lectin (a proximal tubular marker) and *D. biflorus* agglutinin (a collecting duct marker). Interestingly, some of the lining epithelial cells within the cystic structures were positive for *D. biflorus* agglutinin (Figure 5C), but not for *L. tetragonolobus* lectin, which suggests that the cystic pathology originates in the distal collecting ducts. The renal endothelial networks were comparable between *Tsc2* CKO and WT controls (Figure 5C). Some of the tubular epithelial cells were positively stained with vimentin in the *Tsc2* CKO kidney at P21, with significant reduction of Cdh1 expression (Figure 5D). Increased vimentin protein level in the *Tsc2* CKO kidney was verified by Western blot (Figure 5E), suggesting an epithelial-mesenchymal transition as a consequence of *Tsc2* inactivation. In addition, increased apoptotic cells, detected by active caspase 3 immunostaining, were seen in P21 *Tsc2* CKO kidney sections (Figure 5, F and G). These apoptotic cells in the cysts appeared to be shed into the lumens. Interestingly, only minor increase of serum cystatin C was detected in the live *Tsc2* CKO mice around the age of P20 ($10 \pm 3\%$ greater than the littermate WT control; $*P < 0.05$, $n = 5$ pairs), suggesting that kidney function is not severely impaired.

Mesodermal *Tsc2* Deletion Causes Retardation of Pulmonary Alveolar Development

Using mT-mG fluorescence reporter, we confirmed that most lung mesenchymal cells, except pulmonary endothelial

Figure 1 Deletion of *Tsc2* in mesoderm-derived cells resulted in reduced body growth and lethality at early life. **A:** LacZ staining (blue) of embryonic day 14.5 *Dermo1-Cre/Gt(ROSA)26Sor^{tm1Sor}* reporter mice to visualize the pattern of Cre expression. **B:** Comparison of body sizes between *Tsc2^{fx/fx}/Dermo1-Cre* [*Tsc2* conditional knockout (CKO)] mice and their wild-type (WT) littermate controls. **C:** Comparison of whole body weights between *Tsc2* CKO and their littermate controls at different ages. **D:** Altered survival was detected in *Tsc2* CKO mice compared to the WT controls. $n = 4$ per genotype (C); $n = 13$ (4 females and 9 males; D). $*P < 0.05$. P, postnatal day.

Figure 2 Histological comparison of brain (A), liver (B), and heart (C) between *Tsc2^{fx/fx}/Dermo1-Cre* female mice and wild-type (WT) female control mice at postnatal day 14 is shown by hematoxylin and eosin–stained tissue sections. Scale bars: 100 μ m (A and B); 1 mm (C). CKO, conditional knockout.

cells, were targeted by the *Dermo1-Cre* driver during development, whereas no epithelial cells were Cre positive (Figure 6, A and B). The deletion of *Tsc2* in the lung tissue of *Dermo1-Cre/Tsc2^{fx/fx}* mice was then validated by reduced *Tsc2* protein expression and increased mTOR pathway activity (phosphorylation of S6) using Western blot and immunostaining (Figure 6, C and D). Lung development was examined by comparing morphological structures between *Tsc2* CKO mice and their WT littermate controls. Although prenatal development of the lung in *Tsc2* CKO mice was not significantly affected, shown by their lung morphology at P1, significant deficiency of lung alveolarization, which normally occurs from P5 to P30 in mice,²⁴ was detected (Figure 6E). In the *Tsc2* CKO mouse lung, growth of alveolar septa was markedly decreased, which resulted in larger alveolar spaces, within the lungs from P7 to P21. This altered histology was then verified by morphometric measurement of average alveolar size (mean linear intercept) (Figure 6F). Therefore, lung mesenchymal *Tsc2* has an important functional role in promoting lung alveolarization during postnatal development.

To understand the potential mechanisms by which mesenchymal *Tsc2* deletion inhibited lung alveolarization, we analyzed cell proliferation and differentiation. Interestingly, a significant reduction of cell proliferation was

detected in P7 *Tsc2* CKO mouse lungs using nuclear EdU labeling ($6.7 \pm 1.2\%$ in *Tsc2* CKOs versus $11.0 \pm 1.8\%$ in WT controls) (Figure 7, A and B). Differentiation analysis using immunostaining revealed that lung alveolar myofibroblasts in the *Tsc2* CKO mice were markedly reduced, as shown by α -smooth muscle actin–positive cells in alveolar septa (Figure 7C), whereas smooth muscle cells of both airways and vasculatures appeared similar between *Tsc2* CKO and the controls. Interestingly, type II alveolar epithelial cells, shown by Sp-C staining, were also significantly decreased, whereas type I alveolar epithelial cells, shown by T1 α staining, were not changed (Figure 7C). The proximal airway epithelial cells, including ciliated cells and Club cells, shown by β -tubulin IV and CC10 immunostaining, respectively, were comparable between *Tsc2* CKO and WT mice. The number and distribution of alveolar capillary endothelial cells, detected by platelet endothelial cell adhesion molecule 1 staining, appeared to be normal (Figure 7C). Finally, elastin fiber deposition in the tips of alveolar septa and alveolar walls was attenuated in the *Tsc2* CKO lung during alveolar stage (P14) (Figure 7, D and E). Therefore, reduced lung alveolarization in lung mesenchyme-specific *Tsc2* knockout mice is likely caused by both altered cell proliferation and myofibroblast differentiation in the peripheral lung.

Discussion

TSC is a disease affecting multiple organs and systems. Germline homozygous deletion of *Tsc2* in mice results in embryonic lethality, and *Tsc2* heterozygous deletion results in liver, kidney, and lung tumors as the primary phenotypes in adult mice.^{7–9} The manifestations of human TSC include mesenchymal-lineage tumors of the kidney (angiomyolipomas) and lung (lymphangioleiomyomatosis or LAM). To elucidate the consequences of TSC2 inactivation in multiple mesenchymal lineages, we used the *Dermo1*-promoter-Cre that drives Cre expression in mesoderm-derived cells of multiple organs.¹⁵ Herein, we report the novel findings that pan-mesenchymal *Dermo1-Cre*–driven homozygous *Tsc2* CKO mice are viable but have growth retardation, postnatal progressive polycystic kidney disease, and defective alveolarization of the lung.

Mesenchymal cells are heterogeneous, including a variety of smooth muscle cells, endothelial cells, fibroblasts, osteoblasts, and chondrocytes. The specific mesenchymal cell types and mechanisms underlying the reduced body size and weight in our *Tsc2* conditional knockout mice are not known. Increased craniofacial bone mass in neural crest–specific *Tsc1* knockout mice and increased postnatal skeletal bone acquisition in osteoblast-specific *Tsc2* knockout mice have been reported previously.^{25,26} However, retardation of body growth (size or weight) was not found in those prior models. The reduced body size in our mesenchymal knockout model may be the result of aberrant bone development because of loss of

Figure 3 *Dermo1-Cre* driven *Tsc2* deletion in mouse kidney. **A:** *Dermo1-Cre* targeted cells in the kidney were characterized in mT-mG fluorescence protein reporter mice at postnatal day 14 (P14), in which membrane green fluorescent protein (GFP) expression (green) replaced membrane Tomato expression (red) only in cells in which Cre-mediated *loxP* DNA recombination occurred. **B:** *Dermo1-Cre*—mediated *loxP* DNA recombination in mT-mG reporter mice (green) was not detected in P14 kidney epithelial cells (*Cdh1*⁺) or in endothelial cells [platelet endothelial cell adhesion molecule 1 (*PECAM1*)⁺]. **C:** Alterations of *Tsc2* protein and phosphorylation of S6, a downstream target of mTOR pathway, were detected in P7 *Tsc2* conditional knockout (CKO) kidneys by Western blot. **D:** Gross view of kidneys isolated from *Tsc2* CKO mice and wild-type (WT) control mice at P25. Scale bars: 50 μm (**A** and **B**); 1 mm (**D**).

Tsc2 function in both chondrocytes and osteoblasts, and/or the consequence of impaired renal or respiratory function. In our *Dermo1-Cre* mice, Cre is expressed in condensed mesenchyme from which both chondrocytes and osteoblasts are derived during embryonic endochondral ossification (E11.5).¹⁵ At later stages, except in bone marrow cells and osteoclasts, Cre expression in *Dermo1-Cre* mice is also detected in chondrocytes of growth plate cartilage and osteoblasts of the perichondrium, periosteum, and endosteum.¹⁵ A future detailed analysis of *Tsc2* in these targeted cells and skeletal development will be needed to understand the relevant mechanisms underlying the phenotype of retarded body growth.

Previous studies found that deletion of *Tsc1* in smooth muscle cells and cardiomyocytes using an *SM22*-driven Cre resulted in severe biventricular hypertrophy starting from embryonic day 15, with median survival of these *Tsc1* conditional knockout mice of approximately 24 days after birth.^{12,13} Interestingly, modest cystadenoma pathology was also detected in the kidney and thought to be caused by minor leakage of Cre expression into renal epithelial cells. In the lung, no significant changes in alveolarization were reported; instead, significant muscularization of small peripheral pulmonary vessels was noted. In our *Dermo1-Cre*—driven *Tsc2*

knockout mice, no morphological change in heart development was detected (Figure 2), which may be because of lack of cardiomyocyte targeting in our *Dermo1-Cre* driver line (Figure 1). However, in the kidney, our *Tsc2* CKO mice developed severe polycystic pathology within 3 weeks after birth, resulting in destruction of normal kidney structure. In addition, tubular epithelial hyperplasia and epithelial-mesenchymal transition, but no angiomyolipoma-like lesions, appeared by 3 weeks of age. Using the fluorescence reporter to track the *Dermo1-Cre* targeted cells, we found that Cre was expressed specifically in kidney mesenchymal cells, and not in glomerular endothelial cells nor tubular epithelial cells (Figure 3). In addition, overall cell proliferation was increased in the *Tsc2* CKO kidney, consistent with epithelial hyperplasia. The potential mechanisms through which mesenchymal *Tsc2* deletion results in tubular epithelial hyperplasia and cystic change may include a physical blockage of flow by these hyperplastic tubular structures. These findings suggest that *Tsc2* functions within renal mesenchymal cells to maintain normal renal tubular structural homeostasis after development rather than regulating kidney morphogenesis per se.

Kidney lesions occur in most TSC patients and are a common cause of morbidity. These include both

Figure 4 Renal pathology in the *Tsc2* conditional knockout (CKO) mice. **A:** Comparison of overall kidney anatomical structures between the *Tsc2* CKO mice and their littermate controls at different postnatal ages. **B:** Hematoxylin and eosin–stained kidney tissue sections at high magnification. **Black dotted line** highlights a hyperplastic tubule. **C:** Kidney cortex histology shown by periodic acid-Schiff staining. **Arrow** indicates multiple layers of hyperplastic tubular epithelia lining a cystic structure; **arrowhead**, a single layer of epithelium lining a cystic structure. Scale bars: 1 mm (**A**); 50 μ m (**B**); 100 μ m (**C**). P, postnatal day.

mesenchymal and epithelial lesions: mesenchymal angiolipomas and epithelial polycystic kidney disease, oncocytoma, and renal cell carcinoma.^{27,28} The mechanisms of renal cystic disease in TSC, including whether they arise because of epithelial versus mesenchymal inactivation of TSC2, are not yet completely understood. Several findings have focused on an epithelial pathogenesis model, including that TSC2 is required for the membrane

localization of polycystin 1 protein and that TSC2 physically interacts with polycystin 1 to regulate the primary cilium in epithelia.^{29,30} Our data suggest, for the first time, that some of the epithelial cystic lesions in TSC may arise through mesenchymal loss of TSC2 function, supporting a new model for the cyst pathogenesis in TSC. A contiguous gene syndrome, with severe early-onset cystic disease, occurs in human with germline deletions of both *TSC2* and

Figure 5 Cell proliferation and cystic epithelial cells in *Tsc2* conditional knockout (CKO) kidney. **A** and **B**: Proliferative cells were detected by 3-hour 5'-ethynyl-2'-deoxyuridine (EdU) labeling, and the percentage of EdU-positive nuclei (green) was analyzed based on DAPI-nuclear counterstaining (blue). **C**: Endothelial cells in the renal cortex were detected by platelet endothelial cell adhesion molecule 1 (PECAM1) immunostaining. Different tubular epithelial cells were also labeled with *Lotus tetragonolobus* lectin (LTL; green) or *Dolichos biflorus* agglutinin (DBA; red)-specific binding. DBA-positive cells in the cystic epithelia are indicated with an arrow. **D**: Coimmunostaining of Cdh1 (green), vimentin (red), and DBA (gray) for postnatal day 21 (P21) kidneys with indicated *Tsc2* genotypes. Vimentin-positive cells in tubules are indicated with arrowheads. **E**: Increased vimentin in total kidney tissue lysate at P21 was detected by Western blot. β-Actin is a loading control. **F**: Activated caspase 3 immunostaining for apoptotic cells (brown; arrows) in P21 kidneys. **G**: The numbers of apoptotic cells per 100 mm² area of kidney tissue sections are compared. *n* = 3 in each genotype (**B** and **G**). **P* < 0.05. Scale bars = 50 μm (**A**, **C**, **D**, and **F**). WT, wild type.

Figure 6 Lung mesenchyme-specific knockout of *Tsc2* inhibited postnatal alveolarization. **A** and **B**: Lung cells targeted by the *Dermo1-Cre* driver at postnatal day 7 (P7) were analyzed in mT-mG fluorescence reporter mice. **C** and **D**: Altered *Tsc2* protein expression and mTOR activation in P7 *Tsc2* conditional knockout (CKO) lungs were analyzed by Western blot and immunostaining. **E**: Lung histological changes were evaluated by comparing hematoxylin and eosin-stained lung tissue structures between *Tsc2* CKO and wild-type (WT) control at different postnatal ages. **F**: Alveolar sizes were quantitatively analyzed by measuring mean linear intercept. $n = 4$ per genotype (**F**). $*P < 0.05$. Scale bars: 50 μm (**A–D**); 200 μm (**E**). a, airway; GFP, green fluorescent protein; PECAM1, platelet endothelial cell adhesion molecule 1; v, vasculature.

the adjacent polycystic kidney disease 1 (*PKD1*) gene.³¹ More important, specific deletion of *Tsc2* exon 2 to 4 in our model confirms that polycystic renal pathology can be caused by mesenchymal loss of *Tsc2*,¹⁶ without simultaneous deletion of the adjacent *Pkd1* gene,³² which experimentally supports clinical observations that renal cysts can

occur in patients with *TSC1* or *TSC2* mutation but no *PKD1* deletion.³³

In our *Tsc2* CKO mouse lung, a striking reduction of pulmonary alveolarization was observed, without obvious changes in pulmonary vascular and airway smooth muscle cells. Yet, by examining the alveolar septa structure,

Figure 7 Altered lung proliferation and differentiation in *Tsc2* conditional knockout (CKO) mice. **A** and **B**: Proliferative cells were detected by 3-hour 5'-ethynyl-2'-deoxyuridine (EdU) labeling (green) in postnatal day 7 (P7) mice, and quantified based on cell nuclear counterstaining with DAPI (blue). **C**: Lung myofibroblasts/smooth muscle cells, endothelial cells, type II and type I alveolar epithelial cells, airway club cells, and ciliated cells were immunostained with the related protein markers. **D**: Comparison of elastin fibers (black) in lung tissues between *Tsc2* CKO and wild-type (WT) control mice at P7 and P14. **E**: Semiquantification of elastin deposition in P14 lung tissues, measured by the percentage of stained elastin area over total lung tissue area of four tissue sections. * $P < 0.05$. Scale bars = 50 μ m (**A–D**). a, airway; PECAM1, platelet endothelial cell adhesion molecule 1; SMA, α -smooth muscle actin; v, vasculature.

we found that α -smooth muscle actin–positive myofibroblasts in our *Tsc2* CKO lung were significantly reduced, particularly in the tips of the alveolar septa. In addition, overall lung cell proliferation was significantly decreased in the *Tsc2* CKO lung, which is opposite to the

increased cell proliferation detected in the *Tsc2* CKO kidney. Therefore, it appears that loss-of-function mutation in *Tsc2* and subsequent mTORC1 hyperactivation result in strikingly diverse cellular responses depending on cell lineages, organs, and stages of development and

homeostasis. Lung alveolarization occurs postnatally in mice, with the growth of secondary alveolar septa into primary alveolar sacs. Alveolar myofibroblasts play a critical role in driving alveolar septa formation by proliferating and migrating into the tips of the septa and by producing elastin extracellular matrices.³⁴ Defects in alveolar myofibroblast migration to the tip because of abrogation of platelet-derived growth factor signaling results in arrest of pulmonary alveolarization.³⁵ In our *Tsc2* CKO lung, the overall number of alveolar myofibroblasts was significantly reduced, accompanied by decreased elastin deposition within the septa. Therefore, reduced proliferation, differentiation, and function of alveolar myofibroblasts because of loss of *Tsc2* function and the resulting hyperactivation of mTORC1 pathway is one of the pivotal mechanisms underlying the alveolar hypoplastic lung phenotype. In contrast, alveolar capillaries were not significantly altered based on immunostaining, suggesting that alveolar angiogenesis, another driving force for pulmonary alveolarization, may not be a critical *Tsc2*-dependent mechanism for alveolarization. Whether defective alveolarization in these *Tsc2* CKO mice contributes to pulmonary cystic lesions in adult remains unknown, because of their shortened life span. It has been reported that decreased alveolar growth in early life has a negative impact on alveolar homeostasis in an aging population, associated with alveolar destruction and emphysema.³⁶ Therefore, it is possible that reduced alveolarization during lung development may contribute to the pulmonary cystic lesions observed in TSC patients. More important, no LAM-like nodules were detected in the female or male *Tsc2* CKO mice, which died before adulthood. We therefore speculate that the high incidence pulmonary cysts in women with TSC, estimated at 80% by the age of 40 years,³⁷ may reflect both a developmental alveolar defect involving *TSC1/2* inactivation in the lung mesenchyme that is not sex specific, and sex-specific LAM cell-produced proteases and cytokines that mediate tissue destruction. This model could explain the fact that most TSC patients (both male and female) with lung cysts will not progress to symptomatic pulmonary disease. We further speculate that the mesenchymal impact of *TSC2* loss cooperates with LAM cell-produced proteases to enhance the lung destruction. To address these hypotheses, further investigation of the relationship between lung mesenchymal *Tsc2* inactivation and LAM-like pathology is needed.

Acknowledgments

We thank Dr. David M. Ornitz (Washington University, St. Louis, MO) for providing Dermo1-Cre mice; Dr. Michael Gambello (Emory University, Atlanta, GA) for providing floxed-*Tsc2* mice; Dr. Esteban Fernandez (Cell Imaging Core of Children's Hospital Los Angeles) for helping with

confocal imaging; Dr. Kevin Lemley (Children's Hospital Los Angeles) for initial discussion about mouse kidney phenotype; and Dr. Roderick Bronson (Harvard Medical School) for review of the renal pathology.

References

- Curatolo P, Maria BL: Tuberous sclerosis. *Handb Clin Neurol* 2013, 111:323–331
- Henske EP, Jóźwiak S, Kingswood JC, Sampson JR, Thiele EA: Tuberous sclerosis complex. *Nat Rev Dis Primers* 2016, 2:16035
- Hayashi T, Kumasaka T, Mitani K, Yao T, Suda K, Seyama K: Loss of heterozygosity on tuberous sclerosis complex genes in multifocal micronodular pneumocyte hyperplasia. *Mod Pathol* 2010, 23:1251–1260
- Carsillo T, Astrinidis A, Henske EP: Mutations in the tuberous sclerosis complex gene *TSC2* are a cause of sporadic pulmonary lymphangioleiomyomatosis. *Proc Natl Acad Sci U S A* 2000, 97:6085–6090
- Henske EP, McCormack FX: Lymphangioleiomyomatosis: a wolf in sheep's clothing. *J Clin Invest* 2012, 122:3807–3816
- Harari S, Torre O, Moss J: Lymphangioleiomyomatosis: what do we know and what are we looking for? *Eur Respir Rev* 2011, 20:34–44
- Kwiatkowski DJ, Zhang H, Bandura JL, Heiberger KM, Glogauer M, el-Hashemite N, Onda H: A mouse model of *TSC1* reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in *Tsc1* null cells. *Hum Mol Genet* 2002, 11:525–534
- Kobayashi T, Minowa O, Kuno J, Mitani H, Hino O, Noda T: Renal carcinogenesis, hepatic hemangiomatosis, and embryonic lethality caused by a germ-line *Tsc2* mutation in mice. *Cancer Res* 1999, 59:1206–1211
- Onda H, Lueck A, Marks PW, Warren HB, Kwiatkowski DJ: *Tsc2*(+/-) mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background. *J Clin Invest* 1999, 104:687–695
- Crowell B, Hwa Lee G, Nikolaeva I, Dal Pozzo V, D'Arcangelo G: Complex neurological phenotype in mutant mice lacking *Tsc2* in excitatory neurons of the developing forebrain(123). *eNeuro* 2015, 2:ENEURO.0046-15.2015
- Ma A, Wang L, Gao Y, Chang Z, Peng H, Zeng N, Gui YS, Tian X, Li X, Cai B, Zhang H, Xu KF: *Tsc1* deficiency-mediated mTOR hyperactivation in vascular endothelial cells causes angiogenesis defects and embryonic lethality. *Hum Mol Genet* 2014, 23:693–705
- Kayyali US, Larsen CG, Bashiruddin S, Lewandowski SL, Trivedi CM, Warburton RR, Parkhitko AA, Morrison TA, Henske EP, Chekaluk Y, Kwiatkowski DJ, Finlay GA: Targeted deletion of *Tsc1* causes fatal cardiomyocyte hyperplasia independently of afterload. *Cardiovasc Pathol* 2015, 24:80–93
- Malhowski AJ, Hira H, Bashiruddin S, Warburton R, Goto J, Robert B, Kwiatkowski DJ, Finlay GA: Smooth muscle protein-22-mediated deletion of *Tsc1* results in cardiac hypertrophy that is mTORC1-mediated and reversed by rapamycin. *Hum Mol Genet* 2011, 20:1290–1305
- Kwiatkowski DJ: Animal models of lymphangioleiomyomatosis (LAM) and tuberous sclerosis complex (TSC). *Lymphat Res Biol* 2010, 8:51–57
- Yu K, Xu J, Liu Z, Sosic D, Shao J, Olson EN, Towler DA, Ornitz DM: Conditional inactivation of FGF receptor 2 reveals an essential role for FGF signaling in the regulation of osteoblast function and bone growth. *Development* 2003, 130:3063–3074
- Hernandez O, Way S, McKenna J, Gambello MJ: Generation of a conditional disruption of the *Tsc2* gene. *Genesis* 2007, 45:101–106
- Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L: A global double-fluorescent Cre reporter mouse. *Genesis* 2007, 45:593–605
- Soriano P: Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999, 21:70–71
- Chen H, Zhuang F, Liu YH, Xu B, Del Moral P, Deng W, Chai Y, Kolb M, Gaudie J, Warburton D, Moses HL, Shi W: TGF-beta

- receptor II in epithelia versus mesenchyme plays distinct roles in the developing lung. *Eur Respir J* 2008, 32:285–295
20. Sun J, Zhuang FF, Mullersman JE, Chen H, Robertson EJ, Warburton D, Liu YH, Shi W: BMP4 activation and secretion are negatively regulated by an intracellular gremlin-BMP4 interaction. *J Biol Chem* 2006, 281:29349–29356
 21. Luo Y, El Agha E, Turcatel G, Chen H, Chiu J, Warburton D, Bellusci S, Qian BP, Menke DB, Shi W: Mesenchymal adenomatous polyposis coli plays critical and diverse roles in regulating lung development. *BMC Biol* 2015, 13:42
 22. Pajak L, Jin F, Xiao GH, Soonpaa MH, Field LJ, Yeung RS: Sustained cardiomyocyte DNA synthesis in whole embryo cultures lacking the TSC2 gene product. *Am J Physiol* 1997, 273:H1619–H1627
 23. Rennebeck G, Kleymenova EV, Anderson R, Yeung RS, Artzt K, Walker CL: Loss of function of the tuberous sclerosis 2 tumor suppressor gene results in embryonic lethality characterized by disrupted neuroepithelial growth and development. *Proc Natl Acad Sci U S A* 1998, 95:15629–15634
 24. Warburton D, Bellusci S, Del Moral PM, Kaartinen V, Lee M, Tefft D, Shi W: Growth factor signaling in lung morphogenetic centers: automaticity, stereotypy and symmetry. *Respir Res* 2003, 4:5
 25. Fang F, Sun S, Wang L, Guan JL, Giovannini M, Zhu Y, Liu F: Neural crest-specific TSC1 deletion in mice leads to sclerotic craniofacial bone lesion. *J Bone Miner Res* 2015, 30:1195–1205
 26. Riddle RC, Frey JL, Tomlinson RE, Ferron M, Li Y, DiGirolamo DJ, Faugere MC, Hussain MA, Karsenty G, Clemens TL: Tsc2 is a molecular checkpoint controlling osteoblast development and glucose homeostasis. *Mol Cell Biol* 2014, 34:1850–1862
 27. Henske EP: Tuberous sclerosis and the kidney: from mesenchyme to epithelium, and beyond. *Pediatr Nephrol* 2005, 20:854–857
 28. Yang P, Cornejo KM, Sadow PM, Cheng L, Wang M, Xiao Y, Jiang Z, Oliva E, Jozwiak S, Nussbaum RL, Feldman AS, Paul E, Thiele EA, Yu JJ, Henske EP, Kwiatkowski DJ, Young RH, Wu CL: Renal cell carcinoma in tuberous sclerosis complex. *Am J Surg Pathol* 2014, 38:895–909
 29. Kleymenova E, Ibragimov-Beskrovnya O, Kugoh H, Everitt J, Xu H, Kiguchi K, Landes G, Harris P, Walker C: Tuberin-dependent membrane localization of polycystin-1: a functional link between polycystic kidney disease and the TSC2 tumor suppressor gene. *Mol Cell* 2001, 7:823–832
 30. Shillingford JM, Murcia NS, Larson CH, Low SH, Hedgepeth R, Brown N, Flask CA, Novick AC, Goldfarb DA, Kramer-Zucker A, Walz G, Piontek KB, Germino GG, Weimbs T: The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease. *Proc Natl Acad Sci U S A* 2006, 103:5466–5471
 31. Brook-Carter PT, Peral B, Ward CJ, Thompson P, Hughes J, Maheshwar MM, Nellist M, Gamble V, Harris PC, Sampson JR: Deletion of the TSC2 and PKD1 genes associated with severe infantile polycystic kidney disease: a contiguous gene syndrome. *Nat Genet* 1994, 8:328–332
 32. Piontek K, Menezes LF, Garcia-Gonzalez MA, Huso DL, Germino GG: A critical developmental switch defines the kinetics of kidney cyst formation after loss of Pkd1. *Nat Med* 2007, 13:1490–1495
 33. Sampson JR, Maheshwar MM, Aspinwall R, Thompson P, Cheadle JP, Ravine D, Roy S, Haan E, Bernstein J, Harris PC: Renal cystic disease in tuberous sclerosis: role of the polycystic kidney disease 1 gene. *Am J Hum Genet* 1997, 61:843–851
 34. Morrissey EE, Hogan BL: Preparing for the first breath: genetic and cellular mechanisms in lung development. *Dev Cell* 2010, 18:8–23
 35. Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, Pekna M, Hellstrom M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Tornell J, Heath JK, Betsholtz C: PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 1996, 85:863–873
 36. Fletcher C, Peto R: The natural history of chronic airflow obstruction. *Br Med J* 1977, 1:1645–1648
 37. Cudzilo CJ, Szczesniak RD, Brody AS, Rattan MS, Krueger DA, Bissler JJ, Franz DN, McCormack FX, Young LR: Lymphangiogenesis screening in women with tuberous sclerosis. *Chest* 2013, 144:578–585