The Loss of Tuberin Promotes Cell Invasion through the β -Catenin Pathway

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Mutations in the tumor suppressor tuberlin (TSC2) are a common factor in the development of lymphangioleiomyomatosis (LAM). LAM is a cystic lung disease that is characterized by the infiltration of smooth muscle-like cells into the pulmonary parenchyma. The mechanism by which the loss of tuberlin promotes the development of LAM has yet to be elucidated, although several lines of evidence suggest it is due to the metastasis of tuberlin-deficient cells. Here we show that tuberlin-null cells become nonadherent and invasive. These nonadherent cells express cleaved forms of β -catenin. In reporter assays, the β -catenin products are transcriptionally active and promote MMP7 expression. Invasion by the tuberlin-null cells is mediated by MMP7. Examination of LAM tissues shows the expression of cleaved β -catenin products and MMP7 consistent with a model that tuberlin-deficient cells acquire invasive properties through a β -catenin-dependent mechanism, which may underlie the development of LAM.

Keywords: caspase 3; MMP7; LAM; tuberous sclerosis complex

Lymphangioleiomyomatosis (LAM) is a potentially fatal cystic lung disease that occurs almost exclusively in women. There are no proven medical therapies for the treatment of LAM, and the health of patients with LAM deteriorates over an average span of 10 years due to the progressive destruction of the lungs (1). LAM is predominantly caused by tuberlin loss-of-function mutations, forming a genetic link with tuberous sclerosis complex (TSC) (2–4). Recent analyses estimate that 30 to 40% of women with TSC may also be affected with LAM (5).

TSC is a multisystem disease that results in the growth of benign tumors in the brain, kidneys, heart, and skin. It commonly affects the central nervous system, resulting in any combination of disorders including seizures, mental retardation, and behavioral problems (6). One of the most common phenotypic factors for TSC is the development of renal angiomyolipomas (AMLs), which occur in approximately 70 to 80% of patients with TSC (7, 8). AMLs are characterized by the abnormal proliferation of dysplastic vessels and adipose and smooth muscle-like cells. Up to 60% of patients with sporadic LAM are diagnosed with AMLs (9). Genetic analyses have demonstrated that LAM cells and AML cells isolated from patients with LAM contain identical TSC2 mutations, suggesting that tuberlin-deficient AML smooth muscle-like cells may have the potential to migrate and infiltrate the lung (3).

The benign metastasis hypothesis of LAM is further supported by two independent clinical observations. First, after lung transplantation, there have been several case reports in which LAM develops anew in the transplanted donor lung, with

CLINICAL RELEVANCE

Mechanisms underlying lymphangioleiomyomatosis (LAM) remain poorly understood. Our findings provide new insights into the potential contribution of beta-catenin and its effectors, including MMP7, in the pathogenesis of LAM and other disorders affected by the TSC2 pathway.

LAM cells expressing the same TSC2 mutation identified in the native lung (10–13). Second, LAM cells have recently been isolated from the circulatory system and other bodily fluids of patients with LAM, indicating that these migratory cells have the potential to invade a variety of host tissues (14). These lines of evidence strongly suggest that the development of LAM is due to the metastatic spread of tuberlin-deficient cells from other sites of origin.

The mechanism of TSC-related metastasis is unknown. Here, we report that tuberlin-null (*Tsc2*^{−/−}) cells produce nonadherent, viable cells that are invasive. These cells maintain constitutive levels of caspase 3 activity, which promotes the cleavage of β -catenin. Examination of a TSC animal model and human LAM tissues demonstrates the presence of truncated β -catenin, corroborating the significance of cleaved β -catenin in the *in vivo* systems. These truncated forms of β -catenin are transcriptionally active and promote the expression of MMP7, a component of cell invasion. Nonadherent *Tsc2*^{−/−} cells possess the ability to invade collagen matrices and epithelial feeder cell layers in a β -catenin- and MMP7-dependent fashion. We show for the first time that LAM lesions express MMP7, suggesting that the activity of truncated β -catenin is relevant to LAM physiology. These results provide evidence for a role of β -catenin in the invasive phenotype of *Tsc2*^{−/−} cells, which may play an important role in the development of LAM.

MATERIALS AND METHODS

Cell Lines

EEF-4 (*Tsc2*^{+/+}), EEF-8 (*Tsc2*^{−/−}), and EEF-4a (*Tsc2*^{−/−}) are three independently derived fibroblast cell lines from Eker rat embryos (15). LEF2 and ERC18M are two independent *Tsc2*^{−/−} renal epithelial tumor cell lines derived from Eker rats (16). The human embryonic kidney (HEK293T) cell line stably expressing the TOPFLASH reporter was a kind gift of T. Biechele and R.T. Moon (University of Washington).

Animal and Human Tissues

Kidney tumors and adjacent normal kidney tissues were procured from *Tsc2*^{+/−} Eker rats (17). Human lung and LAM tissues were obtained from the National Disease Research Interchange. Experiments involving animal tissues were approved by the Institutional Animal Care and Use Committee, and those involving human tissues were approved by Institutional Review Board, both at the University of Washington.

Cell Viability Assays

Collected cells were rinsed with PBS and resuspended in 1 ml PBS. A total of 100 μ l of cell suspension was added to 500 μ l of 0.4% trypan

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blue solution (Gibco/Invitrogen, Carlsbad, CA), diluted with 400 μ l PBS, and incubated at room temperature for 5 minutes. Viable and nonviable cells were counted by hemacytometer. Each assay was performed in duplicate, and the results shown represent the mean values $\pm$ SEM of three independent experiments, presented as a percentage of viable cells.

Cell Cycle Analyses

Cells were analyzed by 5-bromodeoxyuridine (BrdU) incorporation (18). Confluent *Tsc2*^{-/-} cells (EEF-8, EEF-4a) were incubated with fresh media containing BrdU at a final concentration of 100 mM. Samples were incubated at 37°C for 72 hours after reaching confluence, and nonadherent cells in the media were collected and centrifuged. A total of 2.5×10^5 pelleted cells were then resuspended in Hoechst buffer (0.154 M NaCl, 0.1 M Tris [pH 7.4], 0.1% NP40, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.2% BSA, 1.2 μ g/ml Hoechst 33258 dye [Calbiochem/EMD, Gibbstown, NJ]) for 30 minutes. Cells were counterstained with ethidium bromide and added to a final concentration of 6 μ g/ml for 15 minutes. Chicken erythrocyte nuclei (Riese Enterprises Inc. BioSure Division, Grass Valley, CA) were added to each sample to provide a quantitative numerical standard. The samples were analyzed on a Coulter-epics Elite flow cytometer (Beckman-Coulter, Brea, CA) with approximately 3×10^4 cells counted per sample. Proliferating cells were identified by BrdU quenching of Hoechst fluorescence compared with ethidium bromide fluorescence. DNA content and cell cycle analyses were performed using the software program MultiCycle (Phoenix Flow Systems, San Diego, CA) at the University of Washington Flow Cytometry Facility.

Flow Cytometry Cell Sorting

Samples were analyzed according to the manufacturer's protocol (Annexin V Kit; AbD Serotec, Raleigh, NC) with minor modifications. In brief, nonadherent cells were rinsed in ice-cold PBS and resuspended at a concentration of 5×10^5 cells in 195 μ l of binding buffer. Samples were incubated for 10 minutes in the dark at room temperature with annexin V-FITC reagent. Cells were washed with binding buffer and resuspended in 190 μ l of binding buffer with propidium iodide reagent added before cell sorting analysis. Optimal flow cytometer parameter settings were obtained with cells pretreated with 1 μ M staurosporine (STS) (Calbiochem) or 10 mM NaN₃ for 24 hours to enhance apoptotic and nonviable cell populations, respectively, as controls. Cells were sorted into three distinct populations using an Influx Flow Cytometer (Cytospea/BD, Seattle, WA) at the University of Washington Flow Cytometry Facility.

TUNEL Assays

Apoptosis was detected in tissue sections by TUNEL assays using the In Situ Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN). In brief, tissue sections were rehydrated according to standard protocols. Samples were microwaved in 0.1 M citrate buffer (pH 6.0) for 10 minutes and then rinsed with PBS. Next, samples were incubated with TUNEL reaction mixture in a humidity chamber in the dark at 37°C for 60 minutes and then rinsed with PBS. Converter-peroxidase solution was added to each sample, and samples were incubated in the humidity chamber for 30 minutes and then rinsed with PBS. Samples were incubated with 3,3'-diaminobenzidine (Roche) substrate for 10 minutes, rinsed with PBS, and counterstained with hematoxylin. Mounted samples were examined by light microscopy.

Coimmunoprecipitations

Cell lysates (200 μ g) were incubated overnight at 4°C with 1 μ g of GSK3 β or β -catenin antibodies. Addition of protein A-Sepharose beads (Sigma-Aldrich, St. Louis, MO) with incubation at 4°C for 4 hours followed. Samples were washed with 0.5% NP40 buffer (0.5% NP40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 2.5 mM EDTA, 1 mM NaVO₄, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin) and analyzed by 7 and 10% SDS-PAGE, transferred to immobilon, and immunoblotted for GSK3 β and β -catenin proteins with a 1:1,000 dilution of primary antibody solution (GSK3 β and β -catenin; BD Transduction; Franklin Lakes, NJ). All Westerns were probed with mouse HRP-conjugated secondary antisera (GE Healthcare/Amersham, Pittsburgh, PA) at a dilution of 1:2,000 with proteins detected by enhanced chemiluminescence (ECL) (Amersham).

Tissue Analyses

Eker rat and human lung tissues were homogenized in RIPA buffer (1% NP40, 1% deoxycholic acid, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 25 mM β -glycerophosphate [pH 7.2], 10 mM Tris-HCl pH 7.2, 2 mM EDTA, 50 mM NaVO₄, 240 μ g/ml AEBBSF, 20 μ g/ml aprotinin, 20 μ g/ml leupeptin, 1 μ g/ml pepstatin, 0.4 μ M microcystin) with protein content determined by bicinchoninic acid (BCA) assay. Eker rat (50 μ g), normal lung (100 μ g), and LAM tissues (100 μ g) were analyzed by 7 and 10% SDS-PAGE, transferred to immobilon, and immunoblotted with the indicated antibodies at 1:1,000 dilution (β -actin and smooth muscle α -actin, Sigma; S6 ribosomal protein and phospho-S6 ribosomal protein, Cell Signaling; MMP7, Chemicon). Normal lung tissues were derived at autopsy from donors deceased from nonlung-related illnesses. LAM tissues were derived from explanted lungs of patients with LAM at the time of lung transplantation. Both sets of tissues were obtained from the National Disease Research Interchange (Philadelphia, PA). All Westerns were probed with mouse or rabbit HRP-conjugated secondary antisera (GE Healthcare/Amersham) as appropriate at a dilution of 1:2,000 with proteins detected by ECL.

In Vitro Caspase Assays

Caspase activity assays were performed as described elsewhere with slight modifications (19). Briefly, caspase 3 activity was measured using the substrate DEVD-AMC (BIOMOL). Caspase 6 and 8 activities were measured using the substrates, VEID-AMC and IETD-AMC, respectively (BIO MOL, Plymouth Meeting, PA). Cells were harvested and lysed in 0.5% NP40 buffer. Up to 50 μ g of protein lysate was incubated at 37°C in a caspase assay buffer (200 mM NaCl, 40 mM PIPES [pH 7.2], 20 mM DTT, 2 mM EDTA, 20% sucrose, 0.2% CHAPS, 40 μ M substrate). Enzymatic assays and 7-amino-4-methylcoumarin (Sigma) standard curves were performed in duplicate using a fluorescent plate reader (Packard Instruments) with excitation and emission wavelengths of 360 nm and 460 nm, respectively. Fluorescence of the substrate blank was subtracted as background for each assay. Data analysis was performed using I-Smart software (Packard Instruments). Each activity assay was performed in duplicate, and the results shown represent the mean values $\pm$ SEM of several independent experiments, presented as the pmol of substrate cleaved per mg of protein sample per minute of incubation time.

For the caspase 3 inhibitor assays, *Tsc2*^{-/-} (8) cells before reaching confluence (60% confluent) were treated daily with DMSO (Sigma) or 25 μ M ZVAD-FMK (Calbiochem) for 3 days. During this period, the cells attained confluence. Pooled cells (adherent and nonadherent) from each time point and treatment were harvested, lysed, and analyzed for *in vitro* caspase 3 activity as described above.

Construction of β -Catenin Mutants

The pCS3- β -catenin+MT plasmid was first digested with *Cla*I and *Stu*I enzymes and then relegated to delete the C-terminal Myc tags (MT) generating an untagged *Xenopus* β -catenin construct (pCS3- β -catenin). Three sets of oligos were designed for use in QuikChange mutagenesis techniques (Stratagene, La Jolla, CA). Oligos to delete residues 1 through 115 (caspase 3 cleavage sequence TQFD¹¹⁵) were designed to incorporate a *Bam*HI site followed by a START codon upstream of residue 115. Oligos to delete residues 765 through 781 (caspase 3 cleavage sequence DLMD⁷⁶⁴) were designed to incorporate a STOP codon followed by a *Sna*BI site beginning at residue 765. Oligos to delete residues 752 through 781 (caspase 3 cleavage sequence YPVD⁷⁵¹) were designed to incorporate a STOP codon followed by a *Sna*BI site beginning at residue 752. Gene products were sequenced and then subcloned into the pCS3- β -catenin vector through the use of *Bam*HI and *Sna*BI sites.

Mass Spectrometry Analyses

For identification of cleaved forms of β -catenin in the *Tsc2*^{-/-} cells, N-Adh *Tsc2*^{-/-} cell lysates (1 mg) were immunoprecipitated with β -catenin antibody. Immunoprecipitated products were separated by SDS-PAGE. The gel was fixed overnight in gel fixing solution (50% ethanol, 10% acetic acid) and washed in gel washing solution (50% methanol, 10% acetic acid) before staining with Coomassie Blue R250 (Sigma) stain (0.1% Coomassie Blue, 20% methanol, 10% acetic acid).

for 1 hour. The gel was destained with several washes of destain solution (50% methanol, 10% acetic acid) until background was clear. The gel was equilibrated in storage solution (5% acetic acid) for 1 hour. Coomassie Blue–stained protein bands were excised and subjected to reduction, alkylation, and trypsin digestion procedures as recommended by the University of Washington Medicinal Chemistry Mass Spectrometry Center. Peptide samples were analyzed at the University of Washington Medicinal Chemistry Mass Spectrometry Center by nanoflow LC-MS/MS analysis (Micromass Quattro).

Transfections and Reporter Assays

For transient transfection of β -catenin mutants, HEK293 cells (ATCC) at 50% confluence were transfected with the previously described β -catenin mutant constructs (1.6 μ g) using Lipofectamine PLUS transfection reagent (Invitrogen). Twenty-four hours posttransfection, cells were harvested with PBS and lysed with 0.5% NP40 buffer. Protein content was determined by BCA assay. For immunoprecipitation, 200 μ g of cell lysates were incubated overnight at 4°C with 1 μ g of MMP7 antibody. Addition of protein A-Sepharose beads (Sigma) with incubation overnight at 4°C followed. Samples were washed with 0.5% NP40 buffer. Cell lysate and immunoprecipitated samples were analyzed by 7 and 12.5% SDS-PAGE, transferred to immobilon, and immunoblotted for β -catenin and MMP7 proteins with a 1:1,000 dilution of primary antibody solution. All Westerns were probed with mouse or rabbit HRP-conjugated secondary antisera at a dilution of 1:2,000 with proteins detected by ECL.

For luciferase reporter assays, cDNA expression constructs (0.8 μ g) for β -cat^{WT} (wild-type β -catenin), β -cat^{116–781}, β -cat^{116–764}, β -cat^{116–751}, and dominant negative TCF4 were cotransfected with a renilla luciferase thymidine kinase reporter plasmid (pRLTK) (20 ng) to normalize for transfection efficiencies. 293T-TOPFLASH stable, HEK293, and *Tsc2*^{-/-} (8) cells were used for TOPFLASH, cyclin D1, and MMP7 reporter assays, respectively. A total of 200 ng of the cyclin D1 reporter and 1 μ g of the MMP7 reporter construct (–296 HMT Pro), a kind gift from H. C. Crawford (Stony Brook University), were transfected in each of the appropriate reporter assays. Luciferase activity was determined 24 hours posttransfection using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Each reporter assay was performed in duplicate. The results shown represent the mean values $\pm$ SEM of three independent experiments and are presented as the relative fold increase (relative light units, RLU) over mock-transfected cells (set at a value of 1).

For siRNA transfections, two siRNA constructs targeting rat caspase 3 (NM_012922) were purchased from Ambion (Applied Biosystems/Ambion, Austin, TX). The siRNA sequences are siRNA#1: 5'-CCUUCACUGUGAAGAAAUtt-3' (sense) and 5'-AAUUUCUUCACGAGU AAGGtc-3' (antisense); siRNA#2: 5'-GCAGUUACAAAUGGA UUAAtt-3' (sense) and 5'-UAAUCCAUUUGUAAACUGCgt-3' (antisense). *Tsc2*^{-/-} cells at 20% confluence were transfected with 50 nM of control or caspase 3 siRNA using Lipofectamine PLUS transfection reagent (Invitrogen). Three days posttransfection, samples were treated with 1 μ M STS for 4.5 hours to induce nonadherence. Cells were harvested with PBS and then lysed with 0.5% NP40 buffer. Protein content was determined by BCA assay. Samples were analyzed by 7 and 12.5% SDS-PAGE, transferred to immobilon, and immunoblotted for the indicated proteins with a 1:1,000 dilution of primary antibody solution. All Westerns were probed with mouse or rabbit HRP-conjugated secondary antisera at a dilution of 1:2,000 with proteins detected by ECL.

RT-PCR

Total RNA was isolated from 3×10^7 adherent and nonadherent cells according to the protocol from RNeasy Midi Kit (Qiagen, Valencia, CA). RNA (2 μ g for GAPDH and 3 μ g for MMP7) was subjected to RT-PCR according to the manufacturer's protocol (RETROscript; Ambion) for message analysis. RT-PCR of MMP7 was performed with a nested two-step amplification strategy. The RT reaction was performed with 1.5 mM MgCl₂. RT product (5 μ l) was then subjected to the following initial PCR cycling parameters: one cycle at 95°C for 1 minute, 35 cycles at 94°C for 1 minute, 60°C for 1.5 minute, 72°C for 1 minute, with final extension at 72°C for 5 minutes using external primers. Initial PCR product (10 μ l) was then subjected to the PCR cycling parameters as described pre-

viously using nested primers. Final products were analyzed by 2.0% agarose gel. The primer sequences used were rat GAPDH (414 bp) 5'-TGCATCTGCACCACCAACTGC-3' (sense) and 5'-AATGCCAGC CCCAGCATCAAAG-3' (antisense), external primers for rat MMP7 5'-GGAAAGCTGTCCCCCGTGTTCATG-3' (sense) and 5'-CTCATCC TTGTCAAAGTGAGCATC-3' (antisense), and nested primers for rat MMP7 (244 bp) 5'-GAATTCTCACTAATGCCAAACAGT-3' (sense) and 5'-GTGTTTCTGGCCCATCAAATGGG-3' (antisense).

Invasion Assays

For collagen gel invasion assays, 2 ml of collagen substrate (80% PureCol [INAMED], 10% 10 $\times$ PBS, 9% FBS, 1% PenStrep) were aliquoted per 60-mm plate. Plates were then incubated at 37°C for 24 hours to allow for collagen polymerization. A total of 2.5×10^5 cells were added with 3 ml of DMEM-F12+10% FBS to each plate. Every 2 to 3 days, samples were replenished with fresh DMEM-F12+10% FBS. Pictures were taken at 2 weeks after the addition of cells.

For epithelial feeder cell invasion assays, human epithelial A431 cells (ATCC) were plated at high density onto 60-mm plates containing glass coverslips. After A431 cells had reached confluence, 4×10^5 cells (*Tsc2*^{+/+} [4], *Tsc2*^{-/-} [8], or nonadherent *Tsc2*^{-/-} [8] cells) were added with further incubation at 37°C for 2 to 5 days. Samples were supplemented with fresh DMEM-F12+10% FBS daily. At 2 and 5 days postaddition, cells were fixed with 3% paraformaldehyde in PBS for confocal immunofluorescence analysis. Confocal microscopy was performed using a Leica SP1/MP or a Zeiss 510 META confocal microscope. *Tsc2*^{+/+} and *Tsc2*^{-/-} cells were visualized by the use of smooth muscle actin antisera (Sigma), which does not recognize A431 cells. TOPRO3 (Molecular Probes/Invitrogen) was used to detect the nuclei from all cells. Media from each sample was harvested 2 days after the addition of *Tsc2*^{+/+} or *Tsc2*^{-/-} cells and then examined by MMP7 immunoprecipitation and SDS-PAGE/Western immunoblot analysis for the presence of secreted, active MMP7 (Calbiochem cat# IM47L).

Inhibition of MMP7 activity was performed using two different antibodies targeting MMP7 (Millipore cat# AB8117 and Chemicon cat# MAB13414) (20). Increasing amounts of both antibodies (with each antibody at concentrations of 0, 0.25, and 1.0 μ g/ml) and 1.0×10^5 nonadherent *Tsc2*^{-/-} cells were added simultaneously to collagen coated plates prepared as described previously. Cells were re-fed 24 hours later and then every 2 to 3 days with fresh DMEM-F12+10% FBS. Twenty-four days postaddition, the invasive colony number of each sample was assessed. The results shown represent the average number of collagen-cleared areas counted per field (with a minimum of five fields counted) of two independent experiments.

Statistical Analyses

Statistical analyses were performed with Microsoft Excel 2004 using Student's *t* test.

RESULTS

Tsc2^{-/-} Fibroblasts Exhibit Loss of Contact Inhibition and Proliferate as Nonadherent Cells

To analyze the effects of tuberin on cell proliferation, we compared the growth rates of two independent *Tsc2*^{-/-} rat embryonic fibroblast cell lines (EEF-8, EEF-4a) to an isogenic wild-type rat embryonic fibroblast cell line (EEF-4), all derived from the Eker rat model of TSC (15). In the subconfluent state, the mutant and wild-type cells grew at similar rates (data not shown), but after confluence, the *Tsc2*^{-/-} cells proliferated significantly faster than wild-type cells (rate of proliferation: EEF-8: $3,850 \pm 700$ cells/h, EEF-4a: $3,541 \pm 700$ cells/h; EEF-4: 914 ± 18 cells/h; *P* < 0.05) (Figure 1A). When these samples were subjected to cell cycle kinetic analyses by BrdU-Hoechst flow cytometry (18), the proportion of cells undergoing active cell cycling was significantly higher for the postconfluent *Tsc2*^{-/-} fibroblasts compared with wild-type cells (Figure 1B, right panel). Consistent with the cell proliferation data, no

difference was found between wild-type and mutant cells during the subconfluent period (Figure 1B, left panel). At confluence, the mutant fibroblasts detached from adjacent cells and continued to grow in suspension; this was not observed in the wild-type cells (Figure 1C). By the third day after reaching confluence, more than 25% of the total *Tsc2*^{-/-} cell population was in suspension in the media.

To determine the viability of the detached *Tsc2* mutant cells, trypan blue exclusion assays were performed. The results show that approximately 75% of the nonadherent *Tsc2*^{-/-} (EEF-8 and EEF-4a, herein referred to as 8 and 4a, respectively) cells were found to be viable (Figure 1D). Upon replating and

Figure 1. *Tsc2*-null cells produce a proliferative nonadherent (N-Adh) subpopulation at confluence. (A) After confluence, *Tsc2*^{-/-} cells (EEF-8 [red] and EEF-4a [green]) proliferated faster than wild-type cells (EEF-4 [black]). Cells were manually counted at the indicated time points after confluence. Average error bars ± 0.1 . (B) Cell proliferation as determined by BrdU-Hoechst flow cytometry was analyzed for subconfluent (left graph) and postconfluent (right graph) wild-type (EEF-4 [black]) and *Tsc2* mutant (EEF-8 [red]; EEF-4a [green]) cells over 72 hours. BrdU quenching of Hoechst fluorescence as compared with ethidium bromide fluorescence identified actively cycling cells. The y axis represents the proportion of cells that have passed through the first cell cycle relative to the total number of cells. Average error bars ± 0.1 . (C) Morphology of postconfluent cultures of *Tsc2*^{+/+} and *Tsc2*^{-/-} fibroblasts. Arrows indicate refractile cells that have detached from the plate. (D) N-Adh *Tsc2*^{-/-} cells (8 and 4a) are viable as determined by trypan blue exclusion assays. Each cell viability assay was performed in duplicate, and the results shown represent the mean values $\pm$ SEM of three independent experiments, presented as the percentage of viable cells. In addition, replated N-Adh *Tsc2*^{-/-} cells grow in foci-like colonies (right panels). (E) N-Adh *Tsc2*^{-/-} cells undergo multiple rounds of cell proliferation as determined by BrdU-Hoechst flow cytometry. At confluence, BrdU was added to the media for 72 hours. Nonadherent cells were collected and processed for analyses. Plots depict the number of cells versus Hoechst intensity and show the distribution of cells in G0/G1, G1', and G1" phases representing the relative proportion of cells in the first, second, and third cell cycle postlabeling, respectively.

further incubation for 3 weeks, the nonadherent cells reattached and formed foci-like colonies (Figure 1D, right panels), indicating that these cells remained viable in the nonadherent state and have the ability to reattach and propagate. The nonadherent *Tsc2*^{-/-} cells were further analyzed by BrdU-Hoechst flow cytometry to directly probe their cell cycle activity. Figure 1E shows that these cells incorporated BrdU to quench the fluorescence of the Hoechst dye over three consecutive cell cycles. At 72 hours postconfluence, 74% of EEF-8 and 75% of EEF-4a cells have entered into their second and third cell cycles (indicated by G1' and G1", respectively). These data strongly indicate that the nonadherent *Tsc2*^{-/-} cells are viable and actively growing. Cells lacking functional tuberin exhibit loss of contact inhibition and proliferate as nonadherent cells.

Nonadherent Tuberin-Null Cells Express Truncated β -Catenin

To further characterize the nonadherent *Tsc2*^{-/-} cells, we examined the expression of β -catenin on the basis of our previous studies showing a functional link between the TSC1/TSC2 complex and the Wnt/ β -catenin pathway (21, 22). Upon confluence, wild-type and *Tsc2* mutant fibroblasts up-regulated total β -catenin levels; this differs from our previous observations in epithelial cells where higher β -catenin expression was found in the *Tsc2*-mutant cells, suggesting that a cell-type functional difference may exist between fibroblasts and epithelial cells (Figure 2A; compare lane 4 with lanes 5 and 6). However, we identified faster mobility bands in the nonadherent *Tsc2*^{-/-} cells that were detected by the anti- β -catenin antibody suggestive of truncated fragments (Figure 2A, lanes 7 and 8). To confirm the identity of these bands, cell lysates from the nonadherent *Tsc2* mutant cells were immunoprecipitated with the β -catenin antibody and subjected to mass spectrometry analyses. Sequencing of the tryptic fragments was found to be identical to the peptide sequence of β -catenin. Thus, the faster migrating immunoreactive bands expressed in the nonadherent *Tsc2*^{-/-} cells represent cleaved products of β -catenin.

Figure 2. Viable *Tsc2*-mutant cells express truncated β -catenin. (A) N-Adh *Tsc2*^{-/-} cells (8 and 4a) express truncated forms of β -catenin (upper panel, lanes 7 and 8). Sub = subconfluent; Conf = confluent cell cultures. Expression of β -actin serves as a loading control (lower panel). (B) N-Adh *Tsc2*^{-/-} cells were sorted by flow cytometry technique to isolate annexin V⁻, PI⁻; annexin V⁺, PI⁻; and annexin V⁺, PI⁺ cell populations. Lysates were blotted for β -catenin (upper panel). The Coomassie-stained gel in the lower panel serves as a loading control. The graph represents the cell viability associated with each sorted sample as determined by trypan blue exclusion method. Each cell viability assay was performed in duplicate, and the results shown represent the mean values $\pm$ SEM of three independent experiments, presented as a percentage of viable cells. **P* < 0.01. (C) β -catenin proteins identified in the *Tsc2*-null fibroblasts resemble the forms of β -catenin expressed in renal tumor-derived cell lines ERC18M and LEF2. Upper panel, β -catenin immunoblot; lower panel, β -actin immunoblot; lanes 1 and 2, *Tsc2*^{-/-} (8); lanes 3 and 4, ERC18M; lanes 5 and 6, LEF2 lysate samples. Adh = adherent; N-Adh = nonadherent cell cultures. (D) Kidney tumors from Eker rats overexpress full-length and truncated forms of β -catenin (upper panel, lanes 3, 5, and 7). Middle panel, longer exposure of β -catenin immunoblot. +/+, normal human kidney tissue; +/-, normal kidney tissue (N) from heterozygote rat (Eker rat); -/-, kidney tumor tissue (T) from heterozygote rat (Eker rat). (E) Apoptosis is minimal or undetectable in untreated kidney tumor samples. Representative TUNEL staining from Eker rat kidney tumors. TUNEL-positive apoptotic cells are shown in brown.

Because not all of the nonadherent cells were viable (see Figure 1D), we examined whether the truncated forms of β -catenin identified in the nonadherent *Tsc2*^{-/-} cells originated from a viable or nonviable cell population. These cells were divided into three groups by flow cytometry based on their staining properties with annexin V-FITC and propidium iodide (PI). Early apoptotic cells can be detected through the use of annexin V staining because annexin V has a high affinity for externally localized phosphatidylserine residues that accompany the morphologic changes in the plasma membrane during the early phase of apoptosis. As cells continue to apoptose, nuclear changes occur after the degradation of the plasma membrane, allowing PI to enter the nucleus. Therefore, cells can be sorted into the following fractions: (annexin V⁻, PI⁻), (annexin V⁺, PI⁻), and (annexin V⁺, PI⁺). After sorting, cells were lysed and analyzed for the expression of β -catenin. Only the (annexin V⁻, PI⁻) and (annexin V⁺, PI⁻) nonadherent cell fractions contained truncated β -catenin proteins (Figure 2B, upper panel, lanes 2 and 3). Full-length β -catenin was undetectable in these samples. Truncated forms of β -catenin were not expressed in the (annexin V⁺, PI⁺) cell fraction (Figure 2B, upper panel, lane 4). This latter fraction had approximately 5% of cells that were deemed viable based on trypan blue exclusion assay (Figure 2B, graph). In contrast, the annexin V⁻, PI⁻, and annexin V⁺, PI⁻ cell samples contained 95 and 87% viable cells, respectively. Together, these results demonstrate that the truncated forms of β -catenin originated from the viable, nonadherent *Tsc2*-null cell population and not

from the small pool of “dying” cells under our experimental conditions.

Truncated β -Catenin Is Expressed in Eker Rat Renal Tumors

To further confirm that the expression of truncated β -catenin is not an artifact of the cell lines used, we turned to another set of independently derived *Tsc2*^{-/-} cell lines obtained from the Eker rat model of renal epithelial tumors. The ERC18M and LEF2 renal tumor cells expressed predominantly full-length β -catenin in the adherent phase of their *in vitro* growth (Figure 2C, upper panel, lanes 3 and 5). At confluence, both cell lines exhibited loss of contact inhibition and detached from the plate to grow in suspension similar to the behavior observed in the EEF-4a and EEF-8 *Tsc2*-null fibroblasts (data not shown). The nonadherent ERC18M and LEF2 cells expressed truncated β -catenin of the same molecular mass as that found in the fibroblast lines (Figure 2C, upper panel; compare lanes 4 and 6 with lane 2). Next, we examined paired samples of renal tumors and adjacent nontumor kidney tissues from the Eker rat. Tumor samples from three different animals overexpressed β -catenin protein compared with adjacent normal kidneys (Figure 2D, upper panel, lanes 3, 5, and 7). All kidney tumor samples expressed truncated forms of β -catenin similar in molecular mass to that found in the nonadherent EEF-8 cells (Figure 2D, upper panel; compare lanes 3, 5, and 7 with lane 8). Wild-type kidney expressed predominantly full-length β -catenin (Figure 2D, upper panel, lane 1), whereas *Tsc2*^{+/-} kidneys adjacent to the tumors showed full-length and truncated β -catenin (Figure

2D, middle panel, lanes 2, 4, and 6). The latter observation is likely due to multiple small tumors throughout the heterozygous kidneys. Next, we examined the extent of apoptosis in the primary tumors to determine if apoptotic cells could be the source of truncated β -catenin. Figure 2E shows that untreated kidney tumors had a minimal degree of apoptosis as measured by TUNEL assay. This implies that truncated β -catenin in the tumors is not derived from apoptotic cells but rather is expressed in the viable tumor cells. Together, our *in vitro* observations from four independently derived *Tsc2*-null cell lines plus our *in vivo* data from the Eker rat tumors strongly suggest that the viable *Tsc2*^{-/-} cells are the primary source of truncated β -catenin.

Caspase 3 Activity Contributes to β -Catenin Cleavage

We sought to identify the source of truncated β -catenin in the *Tsc2* mutant cells. Previous reports have demonstrated that β -catenin is cleaved by caspases 3, 6, and 8 (23, 24). To investigate whether one or more of these caspases were responsible for the truncated β -catenin proteins, we first used STS to induce caspase activity in the wild-type cells and compared their β -catenin products with those found in the untreated, nonadherent *Tsc2*^{-/-} cells and with STS-treated, adherent *Tsc2*^{-/-} cells. In wild-type cells, STS treatment led to the appearance of cleaved β -catenin fragments that were indistinguishable in mass to those found in the *Tsc2*-null cells (Figure 3A, upper panel, lane 2). However, the majority of the STS-treated wild-type cells were not viable compared with STS-treated *Tsc2*^{-/-} cells (Figure 3A, graph). These observations highlight the importance of the loss of *Tsc2* in the generation of viable nonadherent cells. Based on *in vitro* caspase assays, STS activated caspase 3 to a much greater extent than caspase 6 or caspase 8 in the *Tsc2*^{+/-} cells (Figure 3B). Similarly, untreated *Tsc2*^{-/-} cells possessed significantly higher levels of caspase 3 activity but not caspase 6 or caspase 8 activity compared with wild-type cells, although the level of caspase 3 activity was much less than that induced by STS treatment in wild-type cells. This activity was maintained in the nonadherent cells (Figure 3C, graph). The level of caspase 3 activity correlated closely with cleaved β -catenin expression. Treatment of the nonadherent *Tsc2*-null cells with a general caspase inhibitor, ZVAD-FMK, prevented the appearance of truncated β -catenin proteins (data not shown), but, more specifically, treatment with two independent caspase 3 siRNA in the *Tsc2*^{-/-} cells significantly reduced the relative amount of cleaved β -catenin products and MMP7 in parallel with the down-regulation of caspase 3 expression (Figure 3D). Together, these results suggest that the loss of *Tsc2* predisposes cells to density-dependent activation of caspase 3, which leads to cleavage of β -catenin while maintaining cell viability.

Cleaved β -Catenin Products Are Transcriptionally Active

Previous work has shown that β -catenin contains five caspase 3 cleavage sites, with three residing within the N-terminus and two within the C-terminus (24). Based on the apparent molecular mass of the cleaved products (~72 and 70 kD) and the mapped caspase 3 cleavage sites, three β -catenin constructs were designed for further examination (Figure 4A; β -cat¹¹⁶⁻⁷⁸¹, β -cat¹¹⁶⁻⁷⁶⁴, and β -cat¹¹⁶⁻⁷⁵¹). By Western immunoblot analysis, Figure 4B shows that two of these deletion constructs (lanes 4 and 5; β -cat¹¹⁶⁻⁷⁶⁴ and β -cat¹¹⁶⁻⁷⁵¹, respectively) resemble the products found in the nonadherent *Tsc2*^{-/-} cells (lanes 6 and 7).

N-terminally truncated forms of β -catenin have been shown to behave in a constitutively active manner because they lack the sites required for binding and phosphorylation by GSK3 β , a key event that regulates β -catenin turnover (25–28). We predicted that the N-terminal deletion of β -catenin by caspase

3 should render the truncated protein active and incapable of interacting with GSK3 β . The β -catenin mutants found in the nonadherent *Tsc2*^{-/-} cells were unable to associate with GSK3 β in coimmunoprecipitation experiments (Figure 4C, upper and lower panels, lanes 2 and 4).

Luciferase reporter assays were performed to address the transcriptional activity of the β -catenin deletion mutants. Results from TOPFLASH assays showed that β -cat¹¹⁶⁻⁷⁶⁴ and β -cat¹¹⁶⁻⁷⁵¹ remained as active as wild-type β -catenin (Figure 4D; compare lanes 4 and 5 with lane 2). Expression of the β -cat¹¹⁶⁻⁷⁸¹ construct showed a substantial increase in TOPFLASH-dependent transcription (Figure 4D, lane 3), as suggested by previous studies (29). Cyclin D1 is a downstream target of β -catenin transcriptional activity (29), and in cyclin D1 reporter assays, β -cat¹¹⁶⁻⁷⁶⁴ and β -cat¹¹⁶⁻⁷⁵¹ retained transcriptional activity toward the cyclin D1 promoter, but at a level lower than that of wild-type β -catenin (Figure 4E; compare lanes 4 and 5 with lane 2). MMP7 is also a downstream target of β -catenin (30), and, in reporter assays using the MMP7 promoter, β -cat¹¹⁶⁻⁷⁶⁴, and to a lesser extent β -cat¹¹⁶⁻⁷⁵¹, maintained considerable transcriptional activity toward the target gene MMP7 and more so than wild-type β -catenin (Figure 4F; compare lanes 4 and 5 with lane 2). This suggests that nonadherent *Tsc2*^{-/-} cells may specifically express MMP7. We used RT-PCR to confirm that MMP7 was actively transcribed in the nonadherent cells. Nonadherent *Tsc2*^{-/-} cells contained substantially more MMP7 mRNA than their adherent counterpart (Figure 4G). Forced expression of β -catenin¹¹⁶⁻⁷⁶⁴, and to a lesser extent β -catenin¹¹⁶⁻⁷⁵¹, led to higher levels of MMP7 protein expression (as 28 kD proenzyme) than wild-type β -catenin and β -catenin¹¹⁶⁻⁷⁸¹ (Figure 4H; compare lanes 4 and 5 with lanes 2 and 3), correlating well with their MMP7 promoter activities. Overall, these results demonstrate that the truncated forms of β -catenin are transcriptionally active and cannot be regulated by GSK3 β . These active β -catenin mutants are responsible for MMP7 expression in the nonadherent *Tsc2*^{-/-} cells.

Nonadherent Tuberin-Null Cells Secrete MMP7 and Are Invasive

Matrix metalloproteinases (MMPs) play an important role in the remodeling of the extracellular matrix during physiological and pathological processes (31). Unregulated degradation of extracellular matrix components facilitates cell invasion. It has become apparent that various MMPs are overexpressed in several pulmonary disorders (32). It has also been demonstrated that MMP2, and to a lesser extent MMP9, are up-regulated in the lungs of patients with LAM (33, 34). MMP7 is one of the few MMPs to be produced by tumor cells and has been shown to be overexpressed in several types of invasive cancers (35). To determine if nonadherent *Tsc2*^{-/-} cells are invasive due to MMP7 production, two types of cell invasion assays were performed. First, collagen gel invasion assays were used because collagen is a substrate for MMP7 (in addition to MMP2 and MMP9) (36). It appeared that nonadherent *Tsc2*^{-/-} cells attached to the substrate as a colony of cells and then invaded and spread throughout the collagen matrix (Figure 5A, right panel). Adherent *Tsc2*^{-/-} cells and adherent *Tsc2*^{+/-} cells were also used in these cell invasion assays. Figure 5A (left panel) depicts a single adherent *Tsc2*^{-/-} cell that was attached to the collagen substrate yet remained incapable of invasion. *Tsc2*^{+/-} cells were also incapable of invading a collagen matrix (data not shown).

Second, epithelial feeder cell invasion assays were used because E-cadherin is a substrate for MMP7 (as opposed to MMP2 and MMP9) (37). When nonadherent *Tsc2*^{-/-} cells were added to an epithelial feeder cell layer, they secreted active MMP7 (cleaved 18 kD protein) into the cell media (Figure 5B,

Figure 3. Caspase 3 activity induces cleavage of β -catenin in $Tsc2$ -null cells. (A) Staurosporine (STS) treatment promotes the cleavage of β -catenin in $Tsc2^{+/+}$ cells. Cells at 90% confluence were treated for 18 hours $\pm$ 1 μ M STS, and cell lysates were processed for immunoblot analysis of β -catenin. Expression of β -actin serves as a loading control (lower panel). Lane 1, untreated $Tsc2^{+/+}$ (4) cells; lane 2, STS-treated $Tsc2^{+/+}$ (4) cells; lane 3, untreated N-Adh $Tsc2^{-/-}$ (8) cells; lane 4, STS-treated Adh $Tsc2^{-/-}$ (8) cells; lane 5, untreated N-Adh $Tsc2^{-/-}$ (4a) cells; lane 6, STS-treated Adh $Tsc2^{-/-}$ (4a) cells. The graph shows the proportion of viable cells as determined by trypan blue exclusion assays in comparison to $Tsc2^{+/+}$ (4) cells treated with STS. Each cell viability assay was performed in duplicate, and the results shown represent the mean values $\pm$ SEM of three independent experiments, presented as a percentage of viable cells. $*P < 0.01$. (B) *In vitro* caspase assays illustrate the effects of STS on caspase 3, 6, and 8 activities in the $Tsc2^{+/+}$ (4) cells. Caspase activities of the $Tsc2^{-/-}$ (8, 4a) cells are also shown for comparison. $*P < 0.05$. (C) $Tsc2^{-/-}$ cells accumulate caspase 3 activity when Conf or N-Adh (graph). $*P < 0.05$. Corresponding immunoblots show the expression of β -catenin (upper panel) and β -actin loading control (lower panel). (D) siRNA mediated depletion of caspase 3 results in the reduction of cleaved β -catenin and MMP7 proteins in $Tsc2^{-/-}$ cells. Cells were transfected with 50 nM of control siRNA (lane 1) or one of two caspase 3 siRNA (lanes 2 and 3). Lysates were examined by immunoblot analysis. Upper panel, caspase 3; upper middle panel, β -catenin; lower middle panel, MMP7; lower panel, β -actin immunoblots. Expression of β -actin serves as a loading control. The graph shows the relative expression of caspase 3, cleaved β -catenin, and MMP7 proteins as compared with full-length β -catenin and β -actin proteins expressed as fold change.

lane 3). The addition of adherent $Tsc2^{-/-}$ or $Tsc2^{+/+}$ cells did not induce the expression of MMP7 into the cell media (Figure 5B, lanes 2 and 4, respectively). Although these cells were able to

attach to the feeder cell layer, they remained unable to invade (data not shown), which is consistent with the data from the collagen gel invasion assays. As visualized by confocal micros-

Figure 4. Cleaved forms of β -catenin are transcriptionally active. (A) Schematic of β -catenin constructs with caspase 3 cleavage sites denoted by black arrowheads. (B) Expression of β -catenin mutants. Lane 1, mock transfection; lane 2, β -cat^{WT}; lane 3, β -cat¹¹⁶⁻⁷⁸¹; lane 4, β -cat¹¹⁶⁻⁷⁶⁴; lane 5, β -cat¹¹⁶⁻⁷⁵¹; lane 6, N-Adh *Tsc2*^{-/-} (8) cells; lane 7, N-Adh *Tsc2*^{-/-} (4a) cells. (C) Coimmunoprecipitation experiments show that truncated β -catenin proteins identified in N-Adh *Tsc2*^{-/-} cells do not associate with GSK3 β (upper and lower panels, lanes 2 and 4). Left panels, GSK3 β immunoprecipitates; right panels, β -catenin immunoprecipitates; upper panels, β -catenin immunoblots; lower panels, GSK3 β immunoblots. (D) TOPFLASH reporter assays demonstrate that β -catenin mutants maintain significant transcriptional activity (compare lanes 4 and 5 with lane 2). (E) Cyclin D1 reporter assays demonstrate that β -catenin mutants maintain some activities (compare lanes 4 and 5 with lane 2). (F) MMP7 reporter assays show that β -catenin mutants are more active than wild-type β -catenin (compare lanes 4 and 5 with lane 2). Lane 1, mock; lane 2, β -cat^{WT}; lane 3, β -cat¹¹⁶⁻⁷⁸¹; lane 4, β -cat¹¹⁶⁻⁷⁶⁴; lane 5, β -cat¹¹⁶⁻⁷⁵¹; lane 6, β -cat^{WT} and dominant negative TCF-4. Each reporter assay was performed in duplicate, and the results shown represent the mean values $\pm$ SEM of three independent experiments, presented as the relative fold increase (relative light units, RLU) over mock-transfected cells (set at a value of 1). * P < 0.05 as compared with mock samples. (G) RT-PCR analysis demonstrates the up-regulation

of MMP7 mRNA in N-Adh *Tsc2*^{-/-} cells (upper panel). Expression of GAPDH serves as a control for RNA expression (lower panel). (H) β -catenin mutant overexpression induces MMP7 protein expression (lanes 4 and 5). HEK293 cells were transfected with the indicated constructs: lane 1, mock transfection; lane 2, β -cat^{WT}; lane 3, β -cat¹¹⁶⁻⁷⁸¹; lane 4, β -cat¹¹⁶⁻⁷⁶⁴; lane 5, β -cat¹¹⁶⁻⁷⁵¹. Cell lysates were immunoprecipitated with MMP7 antibody to detect MMP7 protein (upper panel, lanes 4 and 5). Lower panel, Western immunoblot of IgG bands of MMP7 immunoprecipitated samples serves as a loading control.

copy, nonadherent *Tsc2*^{-/-} cells formed colonies during the invasion process and superficially invaded the top layer of the feeder cells within 2 days (Figure 5B; N-Adh *Tsc2*^{-/-} [2 d]). Three days later, the nonadherent *Tsc2*^{-/-} cells completed traveling through the feeder cells and formed colonies beneath the feeder cell layer (Figure 5B; N-Adh *Tsc2*^{-/-} [5 d]). Together, these results show that the nonadherent *Tsc2*^{-/-} cells have acquired the ability to secrete MMP7 and invade a foreign substrate.

To further investigate the role of caspase 3 in the production of MMP7 proteins, we used siRNA techniques to deplete caspase 3. Down-regulation of caspase 3 expression using two independent siRNA constructs led to a decrease in cleaved β -catenin and MMP7 proteins (Figure 3D, lane 2). These results are consistent with the role of caspase 3 in the expression of truncated forms of β -catenin and its transcriptional target MMP7 in the *Tsc2*^{-/-} cells. To address the requirement for MMP7 in *Tsc2*^{-/-} cell invasion, MMP7 activity was inhibited with the addition of two different functional antibodies specific for MMP7. Both antibodies bind to the active form of MMP7. In collagen gel invasion assays, the addition of these antibodies

resulted in a dose-dependent decrease in invasive colony count (Figure 5C), suggesting that MMP7 activity is necessary to promote *Tsc2*^{-/-}-dependent cell invasion.

LAM Tissues Express Cleaved β -Catenin

To explore the clinical relevance of our findings, we examined the expression of β -catenin and MMP7 in human LAM tissues. Seven samples were obtained from the National Disease Research Interchange without any clinical identifier except for the diagnosis of LAM. Western blot analyses of the tissue lysates are shown in Figure 5D. All LAM tissues have overexpression of β -catenin compared with normal lung, and six of seven samples expressed truncated β -catenin to a variable degree (Figure 5D, upper panel, lanes 3, 4, and 6–9). Five cases each demonstrated expression of MMP7 and smooth muscle actin, whereas phospho-S6, a marker of mTORC1 activity, was present in all LAM but not normal lung tissues. Although some samples do not demonstrate a direct correlation between the observed levels of cleaved β -catenin and MMP7, this likely reflects the vast heterogeneity in tissue sampling and processing.

Figure 5. Nonadherent tuberlin-null cells are invasive. (A) Collagen gel invasion assays show that N-Adh $Tsc2^{-/-}$ cells are invasive (right panel). The white outline depicts the boundary of the collagen gel area cleared by N-Adh $Tsc2^{-/-}$ invasive cells. The left panel depicts an Adh $Tsc2^{-/-}$ cell attached to the collagen matrix (denoted by black arrowhead) without any effects on the underlying collagen gel. The middle panel shows the same cell enlarged 3 $\times$. (B) Feeder cell invasion assays demonstrate that N-Adh $Tsc2^{-/-}$ cells invade epithelial cell layers and secrete cleaved, active MMP7. Lane 3 shows the expression of active MMP7 from immunoprecipitated media at 2 days postaddition. Lane 1, feeder cells; lane 2, feeder cells + Adh $Tsc2^{-/-}$ cells; lane 3, feeder cells + N-Adh $Tsc2^{-/-}$ cells; lane 4, feeder cells + $Tsc2^{+/+}$ cells. Upper confocal images depict a N-Adh $Tsc2^{-/-}$ cell colony invading epithelial cell layers by Day 2 (2 days). Lower images show the continued invasion of N-Adh $Tsc2^{-/-}$ cells through a feeder layer by Day 5 (5 days). Green, smooth muscle actin staining of the N-Adh $Tsc2^{-/-}$ cells; red, nuclei visualized with TOPRO3 in all cells. (C) Inhibition of MMP7 activity blocks the invasion of N-Adh $Tsc2^{-/-}$ cells in col-

lagen gel invasion assays. N-Adh $Tsc2^{-/-}$ cells were incubated with a combination of two MMP7 antibodies, each at the following concentrations: 0, 0.25, 1.0 μ g/ml. The results shown represent the average number of collagen areas cleared by invasive colonies counted per field (with a minimum of five fields counted) of two independent experiments. $*P < 0.05$. (D) Analysis of lymphangioleiomyomatosis tissue samples demonstrates the presence of cleaved β -catenin and MMP7 (lanes 3–9). Immunoblot analyses of lung lysates using the indicated antibodies. Normal lung tissues are represented in lanes 1 and 2.

However, none of the proteins except β -catenin showed truncated fragments to suggest nonspecific tissue degradation. These findings from clinical samples are highly supportive of our *in vitro* data and show for the first time the presence of cleaved β -catenin and MMP7 in human TSC-related pathology. One limitation of our studies is the use of lysates from whole lung tissues that included LAM and non-LAM cells; therefore, the possibility exists for the “bystander” cells as the source of the observed cleaved β -catenin although immunohistochemical analyses suggest a strong correlation between the β -catenin-expressing and smooth muscle actin-expressing LAM cells (data not shown). Future studies using immunohistochemistry-guided microdissection-based proteomic analysis may help to confirm our results.

DISCUSSION

Our study describes a possible mechanism of LAM development involving nonadherent tuberlin-null cells. In this report we show that the $Tsc2^{-/-}$ cells exhibit a loss of contact inhibition, proliferate as nonadherent cells, and display invasive growth behavior. In addition, these cells express truncated forms of β -catenin. These forms of β -catenin are transcriptionally active in reporter assays and are unregulated by GSK3 β (e.g., loss of ability to associate with GSK3 β). β -catenin is found mutated in several types of cancer and particularly in the context of metastatic disease (38). In such cases, the N-terminus of β -catenin is typically found altered because this domain is necessary to regulate β -catenin degradation. The absence of

this domain or mutation of specific residues within this domain results in a stabilized β -catenin protein with equal or greater transcriptional activity than wild-type β -catenin (25–28). One downstream target of mutant β -catenin activity is MMP7, a key component of cell invasion (39). MMP7 is one of the few MMPs produced by tumor cells and found overexpressed in several types of invasive cancers (35, 40). Here we show that nonadherent $Tsc2^{-/-}$ cells secrete MMP7 and prove invasive in collagen and feeder cell invasion assays. Our findings correlate well with a previous study showing that primary LAM cells exhibit increased invasiveness in collagen gel invasion assays (41). Here we are also able to demonstrate that the inhibition of MMP7 activity by antibody incubation substantially decreases the ability of the nonadherent $Tsc2^{-/-}$ cells to invade collagen matrices. Furthermore, the expression of the β -catenin mutant β -cat^{116–764} significantly enhances the transcription of a MMP7 reporter and expression of MMP7 protein, as compared with wild-type β -catenin, demonstrating that nonadherent $Tsc2^{-/-}$ cells become invasive due to cleaved β -catenin activity and the production of MMP7.

Using *in vitro* caspase assays, we found that the induction of caspase activity, in particular caspase 3, promotes the expression of truncated forms of β -catenin. In contrast, the suppression of caspase 3 activity inhibited the expression of truncated forms of β -catenin and MMP7. Caspase 3 activity was found to be increased in the $Tsc2$ -null cells especially under confluent and nonadherent conditions, but these cells remained largely viable. It appears that a limited amount or threshold of caspase

activity is required to initiate the cleavage of β -catenin (~ 20 -fold over basal levels) but without forcing the cells to complete an apoptotic program. It has been suggested that a low constitutive level of caspase activity may be necessary to carry out cellular regulatory processes (42). Our results extend this concept in the *Tsc2*-null cells to acquire new biologic activity (e.g., invasion) through caspase-mediated cleavage of β -catenin. Our findings are consistent with a recent report demonstrating that caspase 3 induces N- and C-terminal cleavage of β -catenin in G401 Wilms' tumor cells. These "floating cells" remained viable, were able to reattach to cell culture plates, and demonstrated a weakened ability for cell aggregation, suggesting enhanced cell motility (43). An unresolved question remains as to why the viable, nonadherent *Tsc2*^{-/-} cells have enhanced caspase 3 activity. One possibility is that mutant β -catenin activity alone allows these cells to escape cell death through some form of feedback mechanism. Alternatively, the activation of the mTOR signaling pathway in the *Tsc2*^{-/-} cells may serve to induce cell survival even in the presence of limited caspase activity. Recently it has been demonstrated that the loss of TSC2 activates the unfolded protein response, a pathway that plays a significant role in the promotion of cell survival during conditions of cellular stress but can also lead to the activation of caspase 3 (44). This avenue of research warrants further investigation.

We showed that the presence of cleaved β -catenin is not an *in vitro* artifact. In a TSC animal model, we found evidence of cleaved β -catenin in spontaneously occurring renal tumors. Eker rat renal tumors and human AMLs are dissimilar in phenotype but are physiologically analogous due to the activation of the mTOR and β -catenin signaling pathways (22, 45). It has been suggested that the nonadherent *Tsc2*^{-/-} cells may originate from tuberin-deficient hamartomas, such as AMLs, and travel to the lung where they invade the lung parenchyma in a "benign metastasis" model (12). Indeed, we provide evidence of cleaved β -catenin and MMP7 expression in human LAM tissues. Combining this with the results of our earlier studies showing the negative regulatory function of TSC1/TSC2 in the canonical Wnt signaling pathway and the aberrant expression of β -catenin targets in LAM (21, 22), the cumulative evidence strongly supports the role of β -catenin in the pathogenesis of LAM. Future studies will address the association between cleaved β -catenin, MMP7, and clinical outcome.

In summary, our observations provide new evidence that the *Tsc2*^{-/-} cells can produce cleaved β -catenin that enhances MMP7 expression to promote cell invasion. These results highlight the potential for targeting the caspase 3 and β -catenin/MMP7 pathways as novel therapeutic approaches in LAM and other TSC-related disorders.

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