Predisposition to tetraploidy in pulmonary vascular smooth muscle cells derived from the Eker rats

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Giu Y, He GH, Walsh MP, Zheng X-L. Predisposition to tetraploidy in pulmonary vascular smooth muscle cells derived from the Eker rats. Am J Physiol Lung Cell Mol Physiol 293: L702–L711, 2007. First published June 15, 2007; doi:10.1152/ajplung.00016.2007.—Somatic mutations in the tuberous sclerosis complex-2 (TSC2) gene are associated with pulmonary lymphangiomyomatosis (LAM), a disorder characterized by benign lesions of smooth muscle and/or smooth muscle-like cells in the lung. However, the cellular mechanisms underlying LAM disease are largely unknown. Given that the TSC2 gene product tuberin is involved in the regulation of cell growth and proliferation, the present study was designed to investigate the potential roles of TSC2 in regulation of the cell cycle. We studied cell cycle profiles of pulmonary vascular smooth muscle cells (SMCs) derived from Eker rats (Tsc2+/−/EK), a genetic model carrying a germline insertion/deletion in one copy of the Tsc2 gene, and the wild-type rats (Tsc2+/+), a noncarrier counterpart. We found that Tsc2+/−/EK but not Tsc2+/+, SMCs displayed increases in cells with ≥4N DNA content (≥4N cells) and in the bromodeoxyuridine (BrdU) incorporation of ≥4N cells. Centrosome number was also increased in Tsc2+/−/EK SMCs, but the mitotic index was comparable between Tsc2+/+ and Tsc2+/−/EK SMCs. Furthermore, Tsc2+/−/EK SMCs showed elevated phosphorylation of p70S6K and increased expression of cell cycle regulatory proteins Cdk1, cyclin B, Cdk2, and cyclin E. Inhibition of the mammalian target of rapamycin (mTOR) pathway by rapamycin not only inhibited the phosphorylation of p70S6K and the expression of cell cycle regulatory proteins but also reduced accumulation of ≥4N cells and BrdU incorporation of ≥4N cells. Therefore, our results demonstrate that Tsc2+/−/EK SMCs are predisposed to undergo tetraploidy, involving activation of the mTOR pathway. These findings suggest an important role of Tsc2 in regulation of the cell cycle.

Tuberous sclerosis complex-2; smooth muscle cell cycle; lymphangiomyomatosis

TUBEROUS SCLEROSIS COMPLEX (TSC) is an autosomal dominant disorder associated with benign hamartomas, which can occur in multiple organs, including brain, kidney, and lung. Genetic studies have demonstrated that somatic mutations in the TSC2 gene are linked to lymphangiomyomatosis (LAM) (4, 9), a disease characterized by benign lesions of smooth muscle and/or smooth muscle-like cells in the lung (42). LAM cells consist of phenotypically heterogeneous groups of smooth muscle cells (SMCs), mainly myofibroblast-like, spindle-shaped, and epithelioid cells (2). Although the origin of LAM cells is unclear, it has been hypothesized that pulmonary LAM cells may be derived from local vascular SMCs that undergo spontaneous mutations within the vessel walls or metastasize from a distant site, such as the kidney (8).

It has been suggested that TSC2, as a tumor suppressor gene, plays critical roles in regulation of cell growth and proliferation. Mice lacking the Tsc2 gene die in midgestation, and the Tsc2 heterozygotes develop cysts and slow-growing tumors in multiple organs (24, 28). Eker rats, which carry a germline insertion/deletion of one copy of the Tsc2 gene, also develop tumors in a variety of tissues (23, 44, 45). Overexpression of Tsc2 in an Eker rat-derived kidney tumor cell line inhibits cell proliferation and suppresses tumorigenicity (21). The roles of TSC2 in regulation of cell growth and proliferation suggest that TSC2 product may be involved in regulation of the cell cycle. Indeed, in whole embryo cultures lacking Tsc2, there was sustained DNA synthesis in cardiomyocytes (32). Antisense inhibition of TSC2 expression induced the entry of quiescent fibroblasts into S phase and prevented cells from arrest at G1/0 in response to serum withdrawal (36). In addition, SMCs derived from lesions of LAM patients showed increased DNA synthesis (13, 14). These studies revealed that the TSC2 gene product might be involved in regulation of S-phase entry. Although it has been shown that loss of tuberin promotes S-phase entry of cells through affecting the stability of p27, a cyclin-dependent kinase inhibitor (37), the mechanisms underlying TSC2 regulation of the cell cycle are still largely unknown.

The TSC2 gene product, tuberin, has been recently linked to the mammalian target of rapamycin (mTOR) pathway, a signaling network involved in the regulation of cell growth and proliferation in response to growth factors and changes in cellular energy levels (25, 33, 34). Tuberin contains a putative GTPase-activating protein (GAP) domain at its COOH terminus, which can suppress the mTOR activity through inhibition of Rheb, a member of the Ras superfamily (11). Loss of tuberin results in activation of mTOR and phosphorylation of its downstream targets, p70S6K and 4E-BP1 (10, 26, 43). Importantly, upregulation of mTOR and activation of S6K have been observed in hamartomas from TSC patients and in SMCs derived from LAM lesions (6, 13). Inhibition of mTOR by rapamycin markedly inhibited phosphorylation of p70S6K and the expression of cell cycle regulatory proteins but also reduced accumulation of ≥4N cells and BrdU incorporation of ≥4N cells. Therefore, our results demonstrate that Tsc2+/−/EK SMCs are predisposed to undergo tetraploidy, involving activation of the mTOR pathway. These findings suggest an important role of Tsc2 in regulation of the cell cycle.

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transition, which requires the interaction of cyclin-dependent kinase-2 (Cdk2) and cyclin E. Interestingly, increased tetraploidy/polyplody of SMCs has been observed in arterial walls of human and animals (1, 12) and found to be associated with aging and hypertension (31). SMC tetraploidization/polyplloidization can be stimulated by angiotensin II in the presence of a mitotic inhibitor (19). The cellular events that promote SMC tetraploidization/polyplloidization involve DNA duplication (endoreduplication) or aberrant mitosis (karyokinesis and/or cytokinesis failure) (16, 17, 29). Furthermore, in some TSC patients, the appearance of giant cells in TSC lesions is a major feature of brain tumors, raising the possibility that TSC is linked to the formation of polyplody, since increased cell size appears to correlate with an increase in polyplody (39). However, studies in Drosophila show that mutations in TSC genes result in increase in size of cells but with normal ploidy (41). Therefore, whether increased polyplody is associated with TSC hamartomas is unclear.

To investigate whether TSC2 is involved in regulation of the smooth muscle cell cycle, we took advantage of the fact that vascular SMCs have the potential to become tetraploid/polyplody and that Eker rats harbor Tsc2 genetic mutation. Cell cycle profiles of pulmonary vascular SMCs derived from Eker rats (Tsc2+/EK) and the wild-type counterparts (Tsc2+/+) were examined. In this article we report that Tsc2+/EK, but not Tsc2+/+, SMCs showed accumulation of cells with 4N DNA content (4N cells) and a fraction of >4N Tsc2+/EK cells continuing DNA synthesis. A potential role of the mTOR pathway in the increased 4N cells was also evaluated.

MATERIALS AND METHODS

Materials. RPMI 1640 medium, fetal calf serum, trypsin-EDTA, and anti-bromodeoxyuridine (BrdU) monoclonal antibody were purchased from Invitrogen (Burlington, ON, Canada). Antibodies against cyclin B, cyclin E, Cdk2, and Cdk1 were obtained from BD Biosciences (Mississauga, ON, Canada), Rapamycin, PD 98059, anti-phospho-S6K, and anti-phospho-MAPK were obtained from Cell Signaling Technology (Beverly, MA). The tissues were then plated in a primary cell culture dish. After culture for 3–5 days, cells grew out from the tissues. When cells reached subconfluence, the tissues were removed and the cells trypsinized for subculture. Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, and the medium was replaced every 2–3 days. The identity of SMCs was confirmed by immunostaining with antibodies against smooth muscle α-actin and h1-calponin. Allele analysis of cultured SMCs was also performed using the PCR method as described. After subculture for 3 days, cells were treated with and without different drugs for the periods of time indicated.

For double thymidine block, cells were incubated with 2 mM thymidine for 20 h, released into fresh medium for 8 h, incubated with 2 mM thymidine for 15 h, and then released into fresh medium. The time point right after release was set as 0 h. Cells were harvested from 0 up to 48 h, as indicated.

Laser scanning cytometer analysis. Cell cycle profiles and BrdU incorporation were analyzed as described previously (18). In brief, cultured cells were labeled with 10 mM BrdU for 60 min before harvesting and fixed in 80 and 100% ethanol. After incubation with 0.1% Triton X-100 and 4 N HCl, cells were immunostained with anti-BrdU antibody and a secondary antibody conjugated to Alexa Fluor-488. The nuclei were counterstained with propidium iodide (PI) in the presence of RNase. Cells were analyzed for their DNA contents and BrdU incorporation with a laser scanning cytometer (LSC).

Immunofluorescence study. Cells grown on coverslips were fixed with 4% paraformaldehyde for 20 min and permeabilized with 100% methanol overnight at −20°C. Cells were then blocked with 2% skim milk for 30 min. Mitotic index analysis was performed as described (17). Briefly, cells were stained with an antibody (1: 300 dilution) against phospho-histone H3 (serine-10), a mitotic marker, and a secondary antibody (1: 400 dilution) conjugated to Alexa Fluor-488. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were inspected with fluorescence microscopy and photographed with a charge-coupled device (CCD) camera. Mitotic cells were identified by the presence of condensed DNA and phosphohistone H3 (serine-10)-positive staining. The mitotic index was calculated as the percentage of mitotic cells vs. total cell count. A minimum of 300 cells was counted on each coverslip.

For observation of centrosomes, anti-γ tubulin was used as a primary antibody. Cells were also double-stained with anti-α-tubulin or anti-PCNA (proliferating cell nuclear antigen) antibody, as indicated. Images were acquired with a laser confocal microscope (Leica DM RXA2) under a ×40 or ×100 oil-immersion lens and photographed with a Cooled Scientific CCD camera (Princeton Instruments, Trenton, NJ). Stacked images collected at 0.5-μm planes were analyzed using Imaris software.

Western blot detection. SMCs grown in 100-mm petri dishes were treated as indicated and lysed with 1 ml of ice-cold lysis buffer. After centrifugation (10,000 × g for 15 min), the supernants were collected for protein measurement using the Bradford assay. Equal amounts of protein samples (80 μg) were separated with 11% SDS-PAGE, followed by transfer to polyvinylidene fluoride (PVDF) membranes. Antibodies against tuberin, Cdk1, Cdk2, cyclin E, cyclin B, p27, phospho-S6K, MAPK, and phospho-MAPK were used as primary antibodies to detect the respective proteins. The secondary antibody was conjugated with horseradish peroxidase (1:3,000 dilution), and peroxidase activity was detected using an ECL detection kit (GE Healthcare, QC, Canada).

Cell sorting. Cultured Tsc2+/EK SMCs were trypsinized and resuspended in 10 ml of PBS, followed by staining with Hoechst 3342 (10 μg/ml) for 10 min. The FACSVantage flow cytometer (Becton Dickinson, equipped with an automated cell deposition unit (ACDU) and
an ultraviolet laser (wavelength = 351 nm), was used to sort cells. Based on the DNA content, cells were sorted into two groups of ≥4N and 2N cells. After sorting, cells were used for PCR and Western blot analysis as described earlier.

Statistical analysis. Results are means ± SE. Statistical comparisons were performed with Student’s t-test for unpaired observations or one-way ANOVA for observations between multiple groups. A P value < 0.05 was considered a significant difference.

RESULTS

SMCs derived from pulmonary arterioles (arteries) of Eker rats exhibit accumulation of cells with ≥4N DNA content. To investigate whether the TSC2 gene affects the smooth muscle cell cycle, we prepared primary cultures of SMCs from pulmonary arteries and arterioles of Eker-carrier rats (Tsc2⁺/EK) and the noncarrier wild-type counterparts (Tsc2⁺/H1) using the explantation method (5). The presence of the Eker insertion in one Tsc2 allele in cultured SMCs was confirmed using PCR analysis. PCR results showed that the wild-type allele dis- one Tsc2 allele in cultured SMCs was confirmed using PCR explantation method (5). The presence of the Eker insertion in one Tsc2 allele in cultured SMCs was confirmed using PCR analysis. PCR results showed that the wild-type allele dis- one Tsc2 allele in cultured SMCs was confirmed using PCR analysis. PCR results showed that the wild-type allele dis-

During routine culture, we observed that Tsc2⁺/EK, but not Tsc2⁺/+, SMCs derived from female rats contained some cells with enlarged nuclei (Fig. 1B, arrows). In addition, cell cycle profiles detected by LSC showed that Tsc2⁺/EK SMCs exhibited a significant increase in cells with ≥4N DNA content (≥4N cells) (Fig. 1D) compared with Tsc2⁺/+ SMCs (Fig. 1C), suggesting the presence of tetraploid cells in SMCs derived from female Eker rats. The accumulation of ≥4N cells in Tsc2⁺/EK SMCs was observed from passages 2 to 8–10. Cells from passages 10–12 or subsequent subcultures did not display a significant increase in ≥4N cells, and cells with apparently normal nuclei became predominant. The increase in ≥4N cells was observed in six of nine female Eker rats but in none of 10 female wild-type rats. In addition, there was no significant difference in cell numbers between Tsc2⁺/EK and Tsc2⁺/+ cells during culture (data not shown). Among nine female Eker rats studied, two developed tumors in their kidneys at the same age (2–3 mo) as other Eker rats. SMCs derived from those two Eker rats did not show accumulation of ≥4N cells but displayed characteristics of senescence during passages 1–3 (data not shown). SMCs from one of nine female Eker rats had cell cycle profiles very similar to those from wild-type rats. In addition, SMCs derived from three of six male Eker rats demonstrated cell cycle profiles similar to those of SMCs derived from male wild-type rats. However, SMCs from the other three male Eker rats (Tsc2⁺/EK) showed a slight increase in ≥4N cells (≈20–25%) compared with those from the wild-type rats (Tsc2⁺/+) (15–18%). Since ≥4N Tsc2⁺/EK SMCs from the male rats were much less than those from the female ones (45–60%), we focused on the observations ac-

Fig. 1. Increased tetraploidy in pulmonary vascular smooth muscle cells (SMCs) derived from Eker rats. A: Tsc2 allele analysis. Pulmonary SMCs derived from Eker (Tsc2⁺/EK) and wild-type (Tsc2⁺/H1) rats were used for DNA extraction and PCR analysis. The wild-type and Eker alleles amplified by PCR show 237- and 183-bp products, respectively. B: morphology of pulmonary vascular SMCs derived from wild-type (left) and Eker rats (right). Cultured SMCs were fixed with ethanol and stained with propidium iodide (PI), followed by inspection using fluorescence microscopy at ×200 magnification. Arrows indicate Tsc2⁺/EK cells with enlarged nuclei. C and D: DNA content analyzed by laser scanning cytometer (LSC). Representative histograms of DNA content (detected as PI integral) vs. cell count in Tsc2⁺/+ (C) and Tsc2⁺/EK SMCs (D). Populations of cells with 2N, 4N, and >4N DNA content are summarized from 5 independent experiments.

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Fig. 2. Cell cycle profiles of Tsc2+/EK SMCs after synchronization. Tsc2+/+ and Tsc2+/EK SMCs were synchronized by double-thymidine treatment as described in MATERIALS AND METHODS. After release from double-thymidine block at the times indicated, cells were fixed and stained with PI in the presence of RNase, followed by LSC analysis to detect the cell cycle distribution based on their DNA contents. The percentages of 4N and >4N cells are summarized from 3 independent experiments.

Fig. 3. Analysis of the mitotic index in Tsc2+/+ and Tsc2+/EK SMCs. Cells cultured in RPMI 1640 medium with 10% FBS for 3 days were fixed with methanol and stained with anti-phospho-histone H3 (serine-10), a mitotic marker, with the nuclei counterstained with DAPI. The mitotic index was expressed as a percentage of mitotic cells vs. total cell count.

4N cells could be in G2 phase, mitotic phase, or tetraploid G1/0. Since cells with enlarged nuclei did not display condensed DNA, it was suggested that 4N cells were not in mitotic phase. To confirm this, cells were stained with an antibody against phospho-histone H3 (serine-10), a mitotic marker, with the nuclei counterstained with DAPI. The mitotic index analysis revealed that both Tsc2+/+ and Tsc2+/EK SMCs had comparable numbers of mitotic cells (n = 4, P > 0.05) (Fig. 3), supporting the conclusion that 4N cells were not the cells arrested in mitotic phase. Thus Tsc2+/EK SMCs may arrest in G2 phase or tetraploid G1/0 phases. Since cells at G2 phase should have two centrosomes, whereas cells at tetraploid G1/0 may have multiple centrosomes (3), we detected centrosome numbers in Tsc2+/EK SMCs. Immunostaining for γ-tubulin, a centrosome component, indicated that 34% of Tsc2+/EK SMCs demonstrated increased numbers of centrosomes (Fig. 4). However, the majority of Tsc2+/+ SMCs contain two or fewer centrosomes. These results suggested that 4N cells could be tetraploid G1/0 cells and Tsc2+/EK SMCs were predisposed to undergo tetraploidization.

Tetraploid Tsc2+/EK cells continue synthesizing DNA. To determine whether tetraploid Tsc2+/EK SMCs indeed entered the next cell cycle, we detected DNA synthesis in 4N cells by labeling cells with BrdU for 60 min under unsynchronized conditions. LSC analysis revealed that the total BrdU incorporation rates were not significantly different between Tsc2+/+ and Tsc2+/EK SMCs (Fig. 5, A and B). However, there was a marked increase in BrdU-positive cells with >4N DNA content in Tsc2+/+ groups compared with Tsc2+/EK SMCs (7.5% vs. 0.5%, n = 5, P < 0.01). The BrdU-positive >4N cells accounted for almost 50% of total BrdU-positive cells in Tsc2+/EK SMCs. The insets in Fig. 5, A and B, represent corresponding BrdU-positive cells with DNA content ≤4N and >4N detected by the relocation feature of LSC. These results suggested that 4N Tsc2+/EK SMCs continued to reduplicate their DNA. To seek more evidence, we further examined whether S-phase cells with enlarged nucleus contained multiple centrosomes by immunostaining cells with antibodies against PCNA and γ-tubulin. The results showed that in Tsc2+/+ SMCs, 12 of 12 PCNA-positive cells examined by confocal microscopy contained one centrosome with one pair of centrioles (Fig. 5C). However, in Tsc2+/EK SMCs, 8 of 12 PCNA-positive cells showed one or two centrosomes, each of which contained four pairs of centrioles (Fig. 5D). These findings indicated that the majority of the 4N cells observed in Tsc2+/EK SMCs was due to the presence of multiple centrosomes, which is characteristic of tetraploid cells. Therefore, we suggest that tetraploidization is a common feature of S-phase cells with enlarged nuclei in Tsc2+/EK SMCs.
that the expression of Cdk1, cyclin B, Cdk2 and cyclin E was of cell cycle regulatory proteins. Western blot analysis showed product in regulation of the cell cycle, we detected expression 4N groups based on their DNA contents. As anticipated, the /H11350 were not significantly different between Tsc2 /H11001). However, the levels of Cdk inhibitors, p27 and p21, (Fig. 6 A (data not shown). To determine whether the mTOR-S6K and/or MAPK pathways were involved in tetraploidization of Tsc2+/EK SMCs, we first examined the effects of rapamycin (an mTOR inhibitor) and PD 98059 (a MAPK kinase inhibitor) on BrdU incorporation rate and the cell cycle profiles. Representative histograms and summarized data (Fig. 7A) show that in Tsc2+/EK SMCs, treatment with rapamycin (50 nM), but not PD 98059 (20 μM), inhibited BrdU incorporation rate, especially in the BrdU-negative >4N cells. Both 4N and >4N cells in Tsc2+/EK SMCs were reduced by treatment with rapamycin compared with the untreated control (28 ± 3.3 and 5 ± 1.9% vs. 38 ± 4.2 and 13 ± 2.3%, respectively) (P < 0.01, n = 5). In contrast, in Tsc2+/+ SMCs, treatment with rapamycin only slightly reduced the cell numbers in S phase (P > 0.05, n = 5). These results suggested that the mTOR pathway, but not the MAPK pathway, was involved in increased tetraploidy in Tsc2+/EK SMCs. To further investigate the involvement of the mTOR-S6K and the MAPK pathways, we analyzed phosphorylation of S6K and MAPK in Tsc2+/+ and Tsc2+/EK SMCs in the absence and presence of either rapamycin or PD 98059. Western blot analysis showed that phosphorylated MAPK and S6K were upregulated in Tsc2+/EK SMCs compared with Tsc2+/+ SMCs. However, the expression of total MAPK or S6K was unchanged. The presence of rapamycin, which inhibited the accumulation of 4N cells and BrdU-positive >4N cells in Tsc2+/+ SMCs (Fig. 7A), markedly reduced phosphorylation of S6K (Fig. 7B), supporting a role for the mTOR pathway in tetraploidization. The presence of PD 98059 inhibited the phosphorylation of MAPK (Fig. 7B) but did not affect the accumulation of >4N cells or BrdU-positive >4N cells (Fig. 7A). Consistently, in Tsc2+/EK SMCs, treatment with rapamycin, but not PD 98059, reduced the increased centrosome numbers (data not shown). To determine whether activation of the mTOR-S6K pathway observed in tetraploidization of Tsc2+/EK SMCs is involved in regulation of cell cycle regulatory proteins, we examined the expression of cyclins and Cdkks in the presence of rapamycin. Western blot analysis showed that treatment with rapamycin, which inhibited both phosphorylation of S6K and tetraploidization of Tsc2+/E K SMCs, prevented the increases in expression of cyclins B and E and Cdkks 1 and 2 in Tsc2+/EK SMCs (Fig. 7C).

**DISCUSSION**

The present study reports that cultured SMCs derived from pulmonary arterioles and arteries of Eker rats (Tsc2+/E K), but

![Image](http://ajplung.physiology.org/download.png)
not the wild-type counterparts (Tsc2+/+), exhibited a spontaneous increase in cells with 4N DNA content. The 4N cells did not contain condensed DNA but had increased centrosomes, suggesting that the cells were at tetraploid stages. In addition, the appearance of BrdU-positive 4N cells and the increase of 4N cells at 12 h that declined at 24 h after release from double thymidine block indicate the cycling of tetraploid cells. Thus Tsc2/EK pulmonary SMCs during primary cell culture are predisposed to tetraploidy/polyploidy, and these tetraploid cells can reenter the cell cycle with 4N DNA contents.

TSC disease is characterized by the occurrence of benign hamartomas in different organs. In the brain, TSC lesions contain characteristic giant cells (22, 35). In the lung, TSC2 mutation-associated LAM disease is characterized by deregulated proliferation of smooth muscle and/or smooth muscle-like cells. However, whether multinucleated SMCs exist in LAM lesions is still unclear. Our observation that predisposition to tetraploidy in pulmonary SMCs derived from Eker rats has revealed the complexity of proliferation properties of Tsc2/EK pulmonary SMCs and their relevance to Tsc2 gene functions. First, polyploidy/aneuploidy is well known to cause genomic instability, leading to deregulated cell proliferation and development of cancer (38). The observation that Tsc2/EK pulmonary SMCs underwent polyploidization raised the possibility that tetraploidy/polyploidy of local pulmonary vascular SMCs could contribute to the formation of LAM cells. In addition, our observation that tetraploidy/polyploidy was more significant in female compared with male Eker rats echoes the fact that LAM disease occurs exclusively in women. However, more detailed investigations are required to confirm whether the observations from in vitro studies reflect the situation in vivo.

Genetic studies indicate that TSC diseases are associated with loss of heterozygosity (LOH) and inactive mutations in one of two TSC genes. In the present study, Tsc2 genes in Tsc2/EK SMCs and sorted 4N Tsc2/EK cells, as detected by PCR, showed one normal allele and one Eker mutant allele, suggesting that tetraploidization/polyploidization of Tsc2/EK SMCs was unlikely to result from LOH. Because of the limitations of the approach used, however, whether any Tsc2 mutations other than Eker mutation or haploinsufficiency lead to tetraploidization/polyploidization in Tsc2/EK SMCs remains to be determined.

Vascular SMCs have the potential to undergo tetraploidization/polyploidization under certain circumstances, as is evident from the increased polyploidy/aneuploidy found in arterial walls of animals and human (1, 12) and associated with aging.

**Fig. 5.** Active DNA synthesis in tetraploid or polyploid Tsc2+/EK cells. A and B: bromodeoxyuridine (BrdU) incorporation of Tsc2+/+ and Tsc2+/EK SMCs detected by LSC. The left and right upper quadrants define BrdU-positive cells with ≤4N and >4N DNA content; insets show nuclear images of BrdU-positive cells with ≤4N and >4N DNA content, respectively, detected by the relocation features of LSC. The numbers of total BrdU-positive and BrdU-positive >4N cells are summarized from 5 independent experiments. C and D: confocal images (taken under ×100 oil objective) of Tsc2+/+ (C) and Tsc2+/EK SMCs (D) stained with PCNA (green), γ-tubulin (red), and DAPI (blue). Images in D show the centrosomes collected at different stacks of 1 Tsc2+/EK cell.
and hypertension (31). In addition, SMC polyploidization can be stimulated by angiotensin II in the presence of a mitotic inhibitor (19). The cellular events resulting in mitotic inhibitor-induced vascular SMC polyploidization involve induction of aberrant mitosis and endoreduplication (16, 17). In Tsc2+/−/EK SMCs, we observed increased tetraploidy/polyploidy without evidence of abnormal mitosis, and a fraction of tetraploid Tsc2+/−/EK cells continued DNA synthesis. These results suggest that tetraploid SMCs reenter the next S phase and undergo cycling. Consistent with the view of tetraploid SMC cycling, it was found that tetraploid SMCs derived from the spontaneous hypertensive rats proliferated in response to growth factors (30).

The molecular mechanisms underlying SMC tetraploidization/polyploidization are unclear. It has been suggested that Akt is involved in promotion of SMC polyploidization, since overexpression of Akt promotes mitotic inhibitor-triggered polyploidization of SMCs (19). Interestingly, it was reported that the mTOR-S6K pathway downstream of Akt was required for endocycling in Drosophila (46). In the present study we found that the mTOR pathway is involved in tetraploidization/
polyploidization in Tsc2+/+ cells, but not the MAPK pathway. A: cell cycle profiles of Tsc2+/+ and Tsc2+/EK cells in the presence of rapamycin (Rapa) and PD 98059. Cultured SMCs, incubated with or without either rapamycin (50 nM) or PD 98059 (20 μM) for 2 days, were fixed and processed for cell cycle analysis by LSC. The number of cells with 2N, 4N, and >4N DNA content and S-phase cells are summarized from 5 experiments. B: Western blot analysis of phospho-S6K, phospho-MAPK, S6K, and total MAPK from Tsc2+/+ and Tsc2+/EK SMCs. Cultured SMCs, treated with or without either rapamycin or PD 98059 for 2 days, were lysed for protein extraction. Protein samples (80 μg of protein from each lysate) were used for Western blot detection with anti-phospho-S6K, anti-phospho-MAPK, and anti-MAPK antibodies, respectively. C: Western blot analysis of cyclin B, cyclin E, Cdk1, and Cdk2 in the same cell lysates as in B. Data are representative of 3 independent experiments with similar results.

Our data are consistent with the findings that upregulation of mTOR and activation of S6K were present in TSC hamartomas from patients and in SMCs derived from LAM tissues (6, 13) and that inhibition of mTOR by rapamycin markedly inhibited phosphorylation of S6K and the increased DNA synthesis in LAM-derived SMCs (13).
The roles of TSC2 in regulation of the cell cycle are largely unknown. Previous studies suggested that TSC2 may be involved in the regulation of S-phase entry (13, 36, 37). The molecular mechanisms underlying TSC2-mediated S-phase entry involve upregulation of G1 cyclin, cyclin D (36), and a decrease in p27 stability (37). Our results showed that the levels of Cdk2 and cyclin E were increased in Tsc2−/EK SMCs compared with Tsc2+/EK SMCs, consistent with the previous finding that loss of TSC2 caused upregulation of G1 cyclins (36). Importantly, our results also showed that the expression of cyclin B was increased in Tsc2−/EK SMCs. Cyclin B was observed in both the nucleus and cytoplasm of Tsc2−/EK SMCs with enlarged nuclei. Furthermore, inhibition of the mTOR-S6K pathway by rapamycin inhibited the accumulation of 4N cells and BrdU-positive >4N cells and also reduced the increased expression of Cdk1, Cdk2, cyclin E, and cyclin B. Given that entry into mitosis requires translocation of cyclin B to the nucleus (40) and that cyclin B has a potential role in the increased expression of Cdk1, Cdk2, cyclin E, and cyclin B. This finding that loss of TSC2 caused upregulation of G1 cyclins (36). Importantly, our results also showed that the expression of cyclin B was increased in Tsc2−/EK SMCs. Cyclin B was observed in both the nucleus and cytoplasm of Tsc2−/EK SMCs with enlarged nuclei. Furthermore, inhibition of the mTOR-S6K pathway by rapamycin inhibited the accumulation of 4N cells and BrdU-positive >4N cells and also reduced the increased expression of Cdk1, Cdk2, cyclin E, and cyclin B. Given that entry into mitosis requires translocation of cyclin B to the nucleus (40) and that cyclin B has a potential role in the promotion of S-phase entry (27), it is reasonable to assume that the increase in expression of cyclin B in 4N Tsc2−/EK cells contribute not only to accumulation of 4N cells but also continuous DNA synthesis without completion of mitosis in 4N cells. Therefore, tetraploidization/polypliodization of Tsc2−/EK SMCs could result from upregulation of cell cycle regulatory proteins through activation of the mTOR-S6K pathway. To our knowledge, this is the first report that tetraploidization/polypliodization occurs in Tsc2−/EK SMCs. Normal SMCs have the potential to undergo tetraploidization/polypliodization in response to various stimuli (1, 12, 19). Tetraploidization/polypliodization of Tsc2−/EK SMCs strongly suggests a stimulatory role of the Eker mutation in cell cycle progression into S phase and upregulation of cell cycle regulatory proteins. The effects of rapamycin have provided additional support for this concept, but more studies are required to elucidate the detailed mechanism.

Our data demonstrate that pulmonary vascular SMCS derived from the Eker rat are predisposed to tetraploidy/polypliody, which involves activation of the mTOR-S6K pathway and upregulation of cell cycle regulatory proteins. This work sheds light on the roles of TSC2 in regulation of the cell cycle.

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