Telomerase Expression Abrogates Rapamycin-Induced Irreversible Growth Arrest of Uterine Fibroid Smooth Muscle Cells

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Abstract

Uterine fibroids are the most common solid tumors found in women of reproductive age. It has been reported that deregulation of the mammalian target of rapamycin (mTOR) pathway plays an important role in the etiology of leiomyoma. Here, we investigated the effect of rapamycin, an inhibitor of mTORC1, on the growth of primary fibroid smooth muscle cells (fSMCs) and human telomerase reverse transcriptase (hTERT)-transduced and immortalized fSMCs. With the primary fSMCs, a 24-hour treatment with rapamycin was sufficient to trigger a growth arrest that was not reversible upon drug removal. By contrast, the growth inhibitory effect of rapamycin on the hTERT-transduced fSMCs was readily reversible, as these cells resumed proliferation upon the withdrawal of the drug. These results suggest that rapamycin-induced irreversible growth arrest of fSMCs is dependent on the senescence barrier that is abrogated by the ectopic expression of telomerase.

Keywords

telomerase, rapamycin, leiomyoma

Introduction

Uterine fibroids are the most common solid tumors found in women of reproductive age. Fibroids have been estimated to affect 75% to 80% of women which is evident from ultrasound examination of the uteri.¹ The annual health care costs related to uterine fibroids are estimated to be in the range of billions of dollars in the United States.²

Most women with symptomatic fibroids undergo hysterectomy, myomectomy, or uterine artery embolization.^{3,4} Nonsurgical treatment of leiomyomas has been primarily through the use of gonadotropin-releasing hormone agonists that suppress circulating levels of estradiol and progesterone.⁵ This treatment option has several adverse side effects such as osteoporosis and menopausal symptoms.⁶

The high prevalence of fibroids in otherwise healthy women suggests that the fibrotic transformation and clonal expansion of fibroid smooth muscle cells (fSMCs) is not a low-frequency event and may therefore involve fewer genetic or epigenetic alterations than those required for cancer development. We have shown that the levels of platelet-derived growth factor (PDGF) C RNA and protein are consistently upregulated in the majority of fibroids when compared to matched adjacent myometrial tissues. We have also shown that exogenous PDGF-CC stimulates, whereas knockdown of endogenous PDGF-RA (the receptor for PDGF-CC) inhibits the clonal expansion of uterine smooth

muscle cells.⁷ The ligand-activated PDGFR tyrosine kinase stimulates a number of intracellular signaling pathways, including the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway. It has been shown that dysregulation of PI3K/AKT leads to the activation of mammalian target of rapamycin (mTOR) in the pathogenesis of leiomyomas.^{8,9} It has been reported that G protein-coupled receptor 10 (GPR10) activates the mTOR pathway to stimulate the proliferation of primary leiomyoma cells in the culture. The aberrant expression of

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GPR10 enhanced fibroid tumor formation in transgenic mice.¹⁰ In the Eker rat model of fibroids, tumor growth is driven by mutational inactivation of the Tsc2 gene, which encodes a negative regulator of mTOR.⁸ A preclinical study was conducted with the Eker rats to test the efficacy of an mTORC1 inhibitor (a rapamycin analog), and the results confirmed that the dysregulated mTOR activity is required as treatment with the mTORC1 inhibitor decreased tumor incidence, multiplicity, and size.⁸

In this study, we examined the effect of rapamycin on the growth of human fSMCs. We have previously shown that human fSMCs have a limited life span (<20 passages) in primary cultures. It has been reported that enforced expression of human telomerase reverse transcriptase (hTERT) can immortalize human myometrium and leiomyoma smooth muscle cells, and the hTERT-immortalized cells exhibited similar morphologic, cellular, and biochemical characteristics as the mortal cells.^{11–13} We therefore generated immortalized fSMCs by ectopic expression of hTERT and then evaluated the effect of rapamycin on the growth of mortal and immortal fSMCs in vitro and in vivo as mouse xenografts in immune-compromised mice.

Materials and Methods

Uterine Tissues

Women who have a hysterectomy because of symptomatic fibroids were recruited to this study. Patients with symptomatic uterine fibroids, who were seen in the Gynecology Outpatient Clinics at the University of Mississippi Medical Center, were considered for participation in the study. The study was approved by local internal review board, and all patients gave written informed consent to participate. Participants completed a questionnaire regarding their reproductive history and their fibroid symptoms that contains parity, hormonal use, ethnicity, last menses, and age. Upon entry into the study, each woman was assigned a unique identifying study number. Matched sets of specimens of normal myometrium and uterine fibroid tissue (1–3 g) were collected in the Operating Room (OR) at the time of hysterectomy, transported to the University of Mississippi Medical Center, Obstetrics and Gynecology research laboratory and cut into small pieces. Specimens were either placed in cryovials and snap frozen or shipped directly to the Moores UCSD Cancer Center for in vitro analysis. Transport medium was Dulbecco modified Eagle medium (DMEM; Mediatech, Inc. Manassas).

Primary Culture of Uterine fSMCs and Lentiviral Transduction

Primary human uterine fSMCs were generated and cultured as described.¹⁴ The primary cultures with passages 2 to 4 derived from 3 different patients were mixed pro rata 1:1:1, and 4×10^6 mixed fSMCs were seeded in one 10-cm culture plate. After one day's culture, the cells were infected with green fluorescent protein (GFP)-Luciferase (LUC) lentiviral stock at 20

multiplicity of infection (MOI) and cultured in a humidified CO₂ (5%) incubator at 37°C. After 24 hours of incubation, the old medium was replaced with fresh complete DMEM, and the cells were cultured for 3 more days before injecting into mice. The methods of lentivirus production and transduction into fSMCs were as described.¹⁴

Mice for Transplantation

RAG2^{-/-}γ_c^{-/-} double knockout (DKO) female mice,¹⁵ 6 to 9 weeks old, were kind gifts from Dr Catriona Jamieson and were bred and maintained on sulfamethoxazole water under specific pathogen-free conditions in the animal care facility at the Moores UCSD Cancer Center. All experiments on mice were conducted in accordance with the guidelines of National Institutes of Health (Bethesda, Maryland) for the care and use of laboratory animals. The animal study protocol (s02013) was approved by UCSD and Medical Experimental Animal Care Committee (United States).

The Transplantation of Human Uterine fSMCs, Rapamycin Treatment, and In Vivo Imaging

The fSMCs infected with lentivirus expressing GFP-LUC were washed once with 10 mL of phosphate-buffered saline (PBS) per 10-cm plate and then trypsinized with 2-mL 0.25% trypsin-EDTA (Invitrogen) for 2 minutes. After aspirating the trypsin solution, 1 mL complete DMEM was added per 10-cm plate to collect the cells. The ~20 mL of cell suspension collected from the 20 plates were combined into one 50-mL sterile conical tube. After counting the cell number, the cell density in the conical tube was adjusted by adding complete DMEM to 4×10^6 cells per mL, and 0.5 mL of this cell suspension containing 2×10^6 cells was then transferred into a 2-mL Eppendorf tube using a 1-mL sterile plastic pipette. We then set up at least 20 Eppendorf tubes, and the content from each tube was used for the implantation of 1 plug. The cells (2×10^6 per tube) were spun down in a Microfuge 22R centrifuge (Beckman Coulter, Fullerton) at 3000 rpm at 4°C for 1 minute. The cell pellet in each Eppendorf tube was resuspended by gentle mixing with 100 μL of ice-cold PBS and then mixed with 100 μL of ice-cold matrigel solution. Each of the 200-μL cell-matrigel mixture was withdrawn into a prechilled 1-mL syringe using a 18-gauge needle; the needle was then changed to a 271/2G one for injection into the flank of a RAG2^{-/-}γ_c^{-/-}/DKO female mouse. To avoid outflow of the cell-matrigel mixture, a heat lamp was used to warm up the mouse skin, thus facilitating the rapid solidification of the matrigel. Each mouse was engrafted with 2 implants at the left and the right flank. On the second day, the first photon flux image was taken with in vivo imaging system (IVIS) before rapamycin or vehicle delivery. Rapamycin was delivered intraperitoneally as a suspension in 10% Tween 80 in 0.9% sodium chloride (NaCl) and a homogeneous suspension was prepared in an agate mortar and pestle (Thomas Scientific, Swedesboro). Rapamycin was given at 5 mg/kg/d. For rescue experiment, rapamycin was just given for

Figure 1. Rapamycin inhibits fibroid smooth muscle cells (fSMCs) proliferation in vitro. A, Different numbers of the green fluorescent protein (GFP)-luciferase (LUC)-transduced fSMCs were seeded in 24-well plates and the luciferase activity was measured by photo flux using in vivo imaging system (IVIS). B, Correlation of fSMC cell number and luciferase photo flux. C, Correlation of fSMC cell number and CCK8 assay. D, Rapamycin dose–response on fSMC proliferation measured by CCK8 assay over the indicated days. E and F, Rapamycin dose–response on fSMC proliferation measured by the IVIS image. G and H, The proliferation of GFP-LUC fSMCs inhibited by different concentrations of rapamycin and measured by CCK8 method (H) and by IVIS (G) after 6 days of treatment. The cell number counted from rapamycin-treated cultures was normalized with that counted from nonrapamycin-treated cultures. I, Rapamycin dose–response on percentage of live fSMCs after 3 or 6 days of treatment measured by Trypan blue assay.

2 consecutive days and followed by vehicle delivery daily afterward. The solution of 10% Tween 80 in 0.9% NaCl was used as vehicle control.

To take IVIS images, whole-body bioluminescence of engrafted mice was detected using the IVIS 200 series (Caliper Inc, Caliper Life Sciences, Hopkinton). Mice were anesthetized with isoflurane using a Gas Anesthesia System and injected intraperitoneally with D-luciferin (150 mg/kg body weight, Caliper Inc.) approximately 2 minutes before imaging. The IVIS imaging was performed every 2 minutes up to about 50 minutes at the exposure time of 60 seconds each to generate a kinetic curve for LUC expression in implants. Mice were subjected to IVIS analysis every 2 days after transplantation. Dorsal images were taken and the total photon flux emission (photons [hv]/s) in the region of interest (ROI), that is, the region containing the matrigel plug, was determined using the Living Image Software (Xenogen, Hopkinton). In the kinetic curve, the linear range of implants of photon flux was from the 8th minute to 26th minute after injection of D-luciferin intraperitoneally. The slope of this linear range is the

LUC activity per unit time, which was used to compare the cell numbers in the implants. See Supplemental Figure S1 for example of the linear regression and slop calculation.

Rapamycin Treatment, Cell Proliferation Assay, and Ex Vivo Imaging

Cell proliferation was measured with the CCK-8 kit (DOJINDO) as described previously.⁷ Briefly, primary fSMCs were seeded in 96-well plates (6000 cells/well) in DMEM containing 10% fetal bovine serum (FBS) and cultured at 37°C, 5% CO₂ for 12 hours. The medium in the well was replaced with 100 μL DMEM containing 10% FBS and indicated doses of rapamycin. The medium containing different doses of rapamycin was changed every day. The CCK-8 assay was performed at indicated time. At days 3 and 6 after rapamycin treatment, the percentage of live cells was determined by Trypan blue exclusion assay. Cell proliferation was also measured by IVIS analysis. The primary fSMCs infected with GFP-LUC lentivirus were

seeded in 24-well plates (60 000 or 40 000 cells/well) and treated with indicated doses of rapamycin in 300 μ L of medium per well following the above-mentioned culture method. To detect the bioluminescent signal of LUC, D-Luciferin was added to the complete medium in 24-well plates at a final concentration of 150 μ g/mL for 5 minutes before imaging with the IVIS analysis for 20 seconds. Images were analyzed using total photon flux emission (photons [hv]/s) in the ROI (the whole well of 24-well plate) by Living Image Software (Xenogen).

Retrovirus-Mediated hTERT Expression in Primary Human Uterine fSMCs

The hTERT retrovirus was kind gift from Dr Fred Levine at the Sanford Burnham Medical Research Institute. The primary cultures of fSMCs at 80% confluency were transduced with the hTERT retrovirus at 10 MOIs in the presence of 8 μ g/mL polybrene for 48 hours and then changed into completed medium supplemented with puromycin (final concentration is 1 μ g/mL). The selection for infected and thus puromycin-resistant cells was for 6 days. The selected cells were cultured in fresh DMEM for 5 days before quantitative real-time polymerase chain reaction (qRT-PCR) and immunoblotting experiments to determine the hTERT overexpression.

RNA Extraction and qRT-PCR

Total RNA from primary cultures of fSMCs was extracted using the RNeasy Mini Kit according to the manufacturer's instructions. The qRT-PCR was performed to measure the hTERT expression level according to our previous method.⁷ The primers for the PCR amplification of hTERT were designed using the Primer Express software (version 2.0). For the hTERT gene, the forward primer is 5'-TGGATGATTC TTGTTGGTGACA-3'; reverse primer is 5'-GCCGCGTTGAAGGTG-3'. For internal control glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene, the forward primer is 5'-GAAGGTGAAGGTCG-GAGTCA-3'; reverse primer is 5'-GACAAGCTTCCCGTTCT-CAG-3'. The relative expression levels of genes were expressed in arbitrary units, where the C_t value of the hTERT gene was normalized to that of GAPDH gene.

Histology and Immunohistochemistry

The UCSD Moores Cancer Center Histology Core performed the hematoxylin and eosin staining and immunohistochemistry staining according to previously established protocols.¹⁴ Antibodies and dilutions used were α -actin (1:150), ribosomal protein S6 (S6; 1:200), phospho-S6 (p-S6; 1:200), phospho-AKT (p-AKT; 1:200), and proliferating cell nuclear antigen (PCNA; 1:200).

Immunoblotting

The human myometrial smooth muscle cells (mSMCs) and fSMCs (either uninfected or infected with hTERT retrovirus

or either untreated or treated with rapamycin) were lysed in lysis buffer (20 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 $\times$ protease cocktail, and 0.55 mmol/L DL-dithiothreitol) and sonicated for 10 to 15 seconds on ice. The lysates were cleared by centrifugation at 21 000g for 15 minutes at 4°C, and the supernatants were quantitated using the Lowry Protein Assay Kit (Bio-Rad Laboratories, Hercules). Whole cell lysates (40 μ g) were fractionated on 4% to 20% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes. The membranes were incubated with anti-hTERT (Calbiochem, Inc. Darmstadt), anti-AKT (Cell Signaling Technology, Inc. Danvers), anti-p-AKT (Cell Signaling Technology, Inc. Danvers), anti-S6 (Cell Signaling Technology, Inc. Danvers), anti-p-S6 (Cell Signaling Technology, Inc. Danvers), and anti-PCNA antibodies (Abcam, Inc. Cambridge), followed by goat antirabbit or antimouse horse raddish peroxidase (HRP)-conjugated secondary antibody and were visualized by chemiluminescence. Stripped membranes were reprobed with anti-GAPDH followed by goat antimouse immunoglobulin G-HRP as loading control.

Statistical Analysis

A 1-tailed, unpaired Student *t*-test was used to analyze statistical differences in proliferation between the SMCs with or without rapamycin treatment. GraphPad Prism 4 package (GraphPad Software) was used for statistical analysis and the paired *t* test was performed, where $P < .05$ was regarded as being statistically significant.

Results

Rapamycin Inhibition of Fibroid Cell Growth

We have previously developed a method to express a GFP-LUC fusion protein in primary fSMCs via retroviral-mediated gene transfer.¹⁴ By seeding varying numbers of GFP-LUC-transduced fSMCs, we could show a linear relationship between cell numbers and the LUC activity, measured by photo flux using a Xenogen imager (Figure 1A and B). When compared with a commercially available cell proliferation assay, CCK8, which measures the metabolic activity, the linearity is better with the luciferase activity (Figure 1B and C). We then treated the fSMCs with different concentrations of rapamycin and measured growth by using the CCK8 and the luciferase activity assays. As shown in Figure 1D, growth of the fSMCs was inhibited by rapamycin at concentrations as low as 0.01 μ g/mL. The rate of growth was slowed in the first 48 hours, and thereafter, the growth was arrested. As the population doubling time of the fSMCs in culture was about 2 days, this growth inhibitory time course suggests that it takes approximately 1 cell cycle time for rapamycin to cause growth arrest. To determine whether the growth inhibition involves increased cell death, we counted the number of live cells by Trypan blue exclusion assay and found no significant increase in cell death in the cultures treated with rapamycin for 3 or 6 days (Figure 1I). Measuring growth inhibition over a range of

Figure 2. Effect of rapamycin on the levels of protein kinase B (AKT), p-AKT, ribosomal protein S6, phosphoribosomal protein S6 (p-S6), and proliferating cell nuclear antigen (PCNA) in 7 fibroid smooth muscle cell (fSMC) primary cultures (P1F, P2F, P3F, P4F, P5F, P6F, and P7F) and 1 myometrial smooth muscle cell (mSMC) primary culture (P5 M). Rapamycin (0.1 mg/mL) treatment was for 2 days.

rapamycin concentrations with both the CCK8 assay and the LUC activity showed the maximal inhibition was reached at 0.01 μ g/mL of rapamycin concentration (Figure 1E-H).

We examined the effect of rapamycin on several different preparations of primary fSMCs, and one preparation of primary mSMCs, and found similar results, that is, 0.01 μ g/mL of rapamycin exerted the maximum growth inhibition of normal mSMCs and fSMCs in cultures (data not shown). Rapamycin is an inhibitor of the mTORC1 kinase complex.¹⁶ Previous studies have established that rapamycin inhibits mTORC1-mediated phosphorylation of ribosomal S6 protein.¹⁷ However, rapamycin also activates the phosphorylation of the AKT kinase.¹⁸ We therefore examined the levels of phospho-AKT and phospho-S6 in fSMCs and mSMCs treated with rapamycin (Figure 2). Consistent with the established effects of rapamycin, we found that the levels of p-S6 decreased, whereas the levels of p-AKT increased after rapamycin treatment, while the levels of S6 and AKT proteins were not significantly affected by rapamycin. Consistent with the inhibition of cell growth, the levels of PCNA (a DNA clamp required for DNA replication) reduced in rapamycin-treated mSMCs and fSMCs, albeit to varying degrees depending on the preparations (Figure 2). Taken together, results in Figures 1 and 2 establish that the *in vitro* growth of mSMCs and fSMCs is sensitive to rapamycin inhibition.

Rapamycin Inhibition of Fibroid Cell Growth in Mouse Xenografts

To determine whether rapamycin can also inhibit the growth of fibroid cells *in vivo*, we constructed fibroid xenografts as subcutaneous matrigel implants in immunocompromised mice and delivered rapamycin systemically through intraperitoneal injection (Figure 3). We monitored the growth and decay of primary fSMCs in these implants with time after transplantation by imaging for LUC activity. To compare the LUC activities among different implants, we measured photo flux as a function of time after the administration of D-luciferin to the anesthetized mice (Supplemental Figure S1). We found that a single time point recording of the photo flux is not an accurate measure of the LUC activities. The variability in photo flux values is likely to be due in part to the

different levels of blood vessel formation in each Matrigel implant. A more accurate measurement of LUC activity, we found, is to determine the photo flux as a function of time after D-luciferin administration. By reading the photo flux every 2 minutes from the 8th minute to 26th minute postinjection of D-luciferin, we calculated photo flux per unit time for each Matrigel implant.

With the primary fSMC xenografts, we typically observed a 2-fold increase in LUC activity during the first 48 hours after implantation, followed by maintenance of LUC activity for up to 6 to 8 days after implantation, followed by a rapid and precipitous reduction in LUC activity (Figure 4A, red line). The decline in LUC activity at days 10 to 12 is associated with cell death, as Matrigel plugs retrieved at this time were found to contain mostly cell debris (data not shown). This life cycle of growth, maintenance, and decay in Matrigel implants has been reproducibly observed in more than 50 implantation experiments with multiple preparations of fSMCs from different patients. At a very low frequency, recovery of cell growth could be observed in some of the Matrigel implants after a few weeks in our previous research. Due to limitations in resources, we did not examine the effect of rapamycin on the rare growth recovery of fSMCs in Matrigel implants in this study.

A typical drug-testing experiment with xenografted mice over a 10-day time course is shown in Figure 4. With rapamycin-treated mice, the initial growth of the fSMCs in the Matrigel implants was not inhibited (Figure 4A, blue line). However, the daily injection with rapamycin eliminated the maintenance phase of the life cycle and hastened the loss of LUC activities (Figure 4A). The shortening of fSMC life cycle by rapamycin *in vivo* is statistically significant and has been verified in multiple independent experiments. Overall, we prepared fSMCs from 10 patient samples and monitored the growth of 12 fibroid xenografts treated with vehicle and 13 treated with rapamycin. We have consistently observed that rapamycin treatment hastened the decay of fSMCs in the Matrigel implants without affecting the initial expansion of these transplanted cells.

We measured the wet weight of implants retrieved at the end of a 2-week time course and found a statistically significant reduction in those implants recovered from rapamycin-treated mice (Figure 4B). We also recovered implants in a shortened time course experiment, where the Matrigel plugs were retrieved at day 7 after implantation when the LUC activities between vehicle and rapamycin-treated xenografts showed the most difference (Figure 4C). At day 7, we found fSMCs expressing α -smooth actin (α -actin) in Matrigel plugs from vehicle or rapamycin-treated mice (Figure 4D and E). These cells contain similar levels of ribosomal protein S6, but the phosphorylation of S6 was reduced in rapamycin-treated implants (Figure 4E).

Rapamycin Induces Irreversible Growth Arrest in fSMCs

Because rapamycin did not induce cell death, it is of interest to determine whether rapamycin-induced growth arrest is reversible. Toward this goal, we devised an experimental strategy where fSMCs were treated with rapamycin for different periods of time in culture, followed by drug removal and culture in regular growth medium (Figure 5A). To avoid the effect of

Figure 3. Schematic diagram of in vivo engraftment of green fluorescent protein (GFP)-luciferase (LUC) fibroid smooth muscle cells (fSMCs) in immune-compromised mice. See Methods section for details.

nutrition depletion of medium on cell viability, we change fresh medium with or without rapamycin every day. We then measured cell viability by 2 methods, IVIS assay for LUC activity or the CCK8 method (Figure 5B). The in vitro results show that the rapamycin-induced growth arrest of cells is not reversible if the cells are treated with 0.01 µg/mL rapamycin for more than 24 hours. We also designed in vivo experimental strategy where fSMCs xenografted mice were treated with rapamycin for 2 days (1 time/1 day) and followed by vehicle delivery every day for other 7 days (Figure 5C). We found that the initial growth of the fSMCs in the Matrigel implants was not inhibited from the IVIS assay results even the cells were underwent 2 days of rapamycin treatment. However, following vehicle delivery could not recover the cells' maintenance phase of the life cycle like vehicle control (Figure 5C, blue line). The in vivo results show that the rapamycin-induced growth arrest of fSMCs in the Matrigel implants was not reversible.

Ectopic Expression of Telomerase Overrides the Irreversible Growth Arrest Induced by Rapamycin in fSMCs

The primary fSMCs or mSMCs isolated from discarded uterine tissues have limited lifespan in culture and in Matrigel implants. To prolong the lifespan of these primary cultured fSMCs or mSMCs, we transduced them with the retrovirus expressing the catalytic subunit of the human telomerase (hTERT). We got 3 hTERT-transduced fSMCs (hTERT-fSMCs) and 1 hTERT-transduced mSMCs (hTERT-mSMCs). Expression of hTERT was confirmed by the measurement of hTERT messenger RNA (Figure 6A) and protein (Figure 6B) in virus-transduced preparations of fSMCs or mSMCs. Importantly, hTERT-fSMCs and hTERT-mSMCs could be expanded more than 50 passages for several months in culture; by contrast, the uninfected parental cells could only be expanded for no more than 20 passages (data not shown). Thus, the ectopic

Figure 4. Rapamycin hastens the in vivo decay of green fluorescent protein (GFP)-luciferase (LUC) fibroid smooth muscle cells (fSMCs) implants in mice. A, In vivo expansion and regression of GFP-Luc fSMC in matrigel implants as a function of time was determined by IVIS imaging, rapamycin was injected intraperitoneally at 5 mg/kg body weight once daily; n = number of implants, each mouse with 2 implants; * $P < .05$. B, Scatter plots of the weight of implants retrieved from mice at the end of 2 weeks after transplantation and treated with vehicle or rapamycin as (A); the mean and the standard error of the mean (SEM) were calculated from 12 vehicle implants and 13 rapamycin-treated implants. C, In vivo expansion and regression of GFP-Luc fSMC in matrigel implants from the xenograft model treated with rapamycin or vehicle for 7 days as (A); the implants were retrieved from mice and hematoxylin and eosin (H&E) staining was performed (magnification 40 $\times$) and (D) immunohistochemistry (IHC; magnification 40 $\times$) with different antibodies that anti- α -actin, ribosomal protein S6 (S6), phosphoribosomal protein S6 (p-S6), phospho-AKT (p-AKT), and proliferating cell nuclear antigen (PCNA).

of hTERT in primary fSMCs or mSMCs is sufficient to “immortalize” these human cells.

We then treated the hTERT-fSMCs or hTERT-mSMCs with rapamycin using the experimental strategy outlined in Figure 5A to determine whether rapamycin can inhibit the growth of hTERT-fSMCs or hTERT-mSMCs and whether this inhibition is irreversible. The cell viability was measured by CCK8 method (Figure 6C). We found that the rapamycin-induced growth arrest is reversible in hTERT-fSMCs to some extent

in both hTERT-mSMCs and hTERT-fSMCs. We also generated xenografted mice with hTERT-fSMCs and treated the mice with rapamycin for 12 days or treated with rapamycin for 2 days (1 time/1 day) and followed by vehicle delivery for other 10 days (Figure 6D). We found that 12 days’ treatment of rapamycin still induced the growth arrest of hTERT-fSMCs in implants (Figure 6D, square line). However, the result from rescue experiment (2 days’ rapamycin treatment and 10 days’ vehicle treatment) shows that the growth arrest

Figure 5. Rapamycin induces irreversible growth arrest of fibroid smooth muscle cells (fSMCs) in vitro and in vivo. **A**, Schematic summary of rapamycin treatment and rescue scheme; the black line shows the time of rapamycin treatment and different color lines show the time of rescue in fresh medium. **B**, Viability of fSMCs was measured with in vivo imaging system (IVIS) imaging or the CCK8 assay; the blue line shows viability of cells at the indicated time (24-168 hours) of rapamycin treatment, the red line shows viability of cells at 168 hours and each point indicates the total time in rapamycin, for example, the 24-hour point on the red line shows viability of cells treated for 24 hours with rapamycin followed by 144 hours of rescue in fresh medium (old medium was replaced with fresh medium containing or not containing rapamycin every day). **C**, In vivo rapamycin rescue experiment, the transplanted mice were treated with rapamycin for 2 days as described in Figure 4 and followed by vehicle treatment for additional 7 days; red arrow points the day of stopping rapamycin treatment; n = number of implants; * indicates $P < .05$.

of hTERT-fSMCs in implants is reversible (Figure 6D, triangle line). There is not maintenance phase of life cycle in hTERT-mSMCs in implants with or without rapamycin treatment (Figure 6E).

Discussion

As the inhibitor of mTOR signaling, rapamycin and its analogs have already been in clinical trials for several conditions such as cancers and cardiovascular diseases.^{19,20} A problem of rapamycin-based therapy is the wide disparity in the dose for inhibiting mTOR signaling under different conditions.²¹ A previous study with the Eker rat fibroid model has shown a rapamycin analog can inhibit fibroid cell growth in vitro and in the rats.⁸ In this study, we examined the effect of rapamycin on the growth of primary human fSMCs in vitro and in vivo. Our

results showed that rapamycin inhibited fSMCs growth at low concentration (0.01 $\mu\text{g/mL}$) but did not increase cell death even at high concentration (10 $\mu\text{g/mL}$; Figure 1 and 2). Rapamycin also inhibited the growth of fSMCs in xenografts implanted in immunocompromised mice (Figure 4). Interestingly, growth inhibition caused by rapamycin was found to be irreversible with the primary fSMCs (Figure 5). However, rapamycin did not induce a stable growth arrest with the hTERT-immortalized fSMCs (Figure 6). These results suggest that rapamycin-induced growth arrest requires the senescence barrier that can be abrogated by the maintenance of telomeres.

There has been evidence pointing to the clonal origin of leiomyoma.²² A recent research showed that the “side population” cells isolated from human leiomyoma were putative tumor-initiating cells or fibroid stem cells.²³ The expression of hTERT and the ability to maintain telomere length is an

Figure 6. Rapamycin induces reversible growth arrest of human telomerase reverse transcriptase (hTERT)-fibroid smooth muscle cells (fSMCs) in vitro and in vivo. A, The hTERT expression level of hTERT-fSMCs was examined by quantitative real-time polymerase chain reaction (qRT-PCR) and (B) by Western blot assay; au indicates arbitrary units. C, The hTERT-fSMCs or hTERT-mSMCs viability of rapamycin rescue assay was measured with CCK8 method, the diamond line shows viability of cells after 24 to 168 hours of rapamycin treatment, the square line shows viability of cells treated with indicated time with rapamycin, and then rescued with fresh medium for the rest time of 168 hours. D, Rapamycin rescue assay of green fluorescent protein (GFP)-luciferase (LUC)-transduced hTERT-fSMCs and (E) hTERT-mSMCs in vivo was measured by IVIS; the triangle line in (D) indicates that the xenografted mice were treated with rapamycin for 2 days and followed by vehicle delivery for other 7 days; arrow points the day of stopping rapamycin treatment; red line means that the xenografted mice were treated with rapamycin for 12 days in (D) or 11 days in (E); n = number of implants.

important attribute of stem cells.^{24,25} Furthermore, the contribution of telomerase activity and telomere maintenance to chemotherapy resistance has been reported.²⁶ Interestingly, we found that rapamycin could not induce irreversible growth arrest in hTERT-immortalized fSMCs. Because the senescence barrier may be activated by rapamycin to arrest fSMC growth, breaching that barrier, for example, in the fibroid stem cells, would prevent rapamycin from inducing a lasting growth arrest.

As we previously reported, human fSMC growth could be sustained over weeks to form fibroid-like tissues in the transplanted mice.¹⁴ This long-term fibroid growth in transplanted mice was observed with only a few of the fMSC preparations. However, with the majority of fSMC preparations, a robust in vivo expansion is invariably followed by a rapid decline within 8 to 12 days following transplantation. To determine whether the decay of fSMC growth in vivo is due to the intrinsic limitation of the life span of these primary fSMCs, we generated immortalized fSMCs

by the enforced expression of hTERT that maintains and/or lengthens the telomere ends to override replicative senescence. As has been previously reported,¹¹⁻¹³ we were able to immortalize fSMCs and mSMCs by retrovirus-mediated ectopic hTERT expression. Although hTERT expression prolonged the lifespan of fSMCs and mSMCs to more than 50 passages in culture, it was unable to prevent the decay of cell growth as xenografts in the transplanted mice. Our finding that immortalization is not sufficient to sustain the growth of fSMC xenografts suggests that sustained growth in mouse xenografts requires factors other than immortalization. The consistent long-term growth of human fibroid xenografts in mice will likely to require identification and sorting of fibroid tumor-initiating cells.

Authors' Note

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Declaration of Conflicting Interests

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Supplemental Material

Supplemental Figure S1 is available at <http://rs.sagepub.com/supplemental>.

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