



Published in final edited form as:

*Otolaryngol Head Neck Surg.* 2015 May ; 152(5): 881–888. doi:10.1177/0194599815573708.

## Rapamycin Inhibits Human Laryngotracheal Stenosis–derived Fibroblast Proliferation, Metabolism, and Function in Vitro

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### Abstract

**Objective**—To determine if rapamycin inhibits the growth, function, and metabolism of human laryngotracheal stenosis (LTS)–derived fibroblasts.

**Study Design**—Controlled in vitro study.

**Setting**—Tertiary care hospital in a research university.

**Subjects and Methods**—Fibroblasts isolated from biopsies of 5 patients with laryngotracheal stenosis were cultured. Cell proliferation, histology, gene expression, and cellular metabolism of LTS-derived fibroblasts were assessed in 4 conditions: (1) fibroblast growth medium, (2) fibroblast growth medium with dimethylsulfoxide (DMSO), (3) fibroblast growth medium with  $10^{-10}$  M (low-dose) rapamycin dissolved in DMSO, and (4) fibroblast growth medium with  $10^{-9}$  M (high-dose) rapamycin dissolved in DMSO.

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#### Disclosures

**Competing interests:** None.

**Sponsorships:** None.

**Funding source:** None.

No sponsorships or competing interests have been disclosed for this article.

#### Author Contributions

**Daryan R. Namba**, acquisition, drafting, approval, agreement; **Garret Ma**, acquisition, revising, approval, agreement; **Idris Samad**, acquisition, revising, approval, agreement; **Dacheng Ding**, acquisition, revising, approval, agreement; **Vinciya Pandian**, acquisition, revising, approval, agreement; **Jonathan D. Powell**, concept, revising, approval, agreement; **Maureen R. Horton**, concept, revising, approval, agreement; **Alexander T. Hillel**, concept, drafting, approval, agreement.

**Results**—The LTS fibroblast count and DNA concentration were reduced after treatment with high-dose rapamycin compared to DMSO ( $P = .0007$ ) and normal ( $P = .0007$ ) controls. Collagen I expression decreased after treatment with high-dose rapamycin versus control ( $P = .0051$ ) and DMSO ( $P = .0093$ ) controls. Maximal respiration decreased to 68.6 pMoles of oxygen/min/10 mg/protein from 96.9 for DMSO ( $P = .0002$ ) and 97.0 for normal ( $P = .0022$ ) controls. Adenosine triphosphate (ATP) production decreased to 66.8 pMoles from 88.1 for DMSO ( $P = .0006$ ) and 83.3 for normal ( $P = .0003$ ) controls. Basal respiration decreased to 78.6 pMoles from 108 for DMSO ( $P = .0002$ ) and 101 for normal ( $P = .0014$ ) controls.

**Conclusions**—Rapamycin demonstrated an anti-fibroblast effect by significantly reducing the proliferation, metabolism, and collagen deposition of human LTS fibroblast in vitro. Rapamycin significantly decreased oxidative phosphorylation of LTS fibroblasts, suggesting a potential mechanism for the reduced proliferation and differentiation. Furthermore, rapamycin's anti-fibroblast effects indicate a promising adjuvant therapy for the treatment of laryngotracheal stenosis.

### Keywords

human; trachea; laryngotracheal stenosis; rapamycin; fibroblasts; collagen

### Introduction

Laryngotracheal stenosis (LTS) is a condition characterized by a buildup of pathologic fibrotic tissue in the airway leading to a narrowing in the tracheal opening. LTS can adversely affect voice and breathing and can progress to life-threatening airway compromise, necessitating tracheostomy or multiple surgical procedures.<sup>1</sup> LTS has multiple etiologies, including iatrogenic tracheostomy tube trauma or long-term endotracheal tube intubation, autoimmune disease, radiation, and idiopathic.<sup>1-4</sup> It has been hypothesized that LTS may be propagated by an abnormal immune response in which injury to the epithelium and lamina propria of the subglottis and trachea spurs subepithelial leukocyte activity, edema, and chronic inflammation.<sup>5,6</sup> The sustained inflammation results in fibroblast proliferation, collagen deposition, and ultimately the formation of scar tissue in the airway.<sup>5-7</sup>

Current medical therapies are not successful in halting the development of laryngotracheal scar tissue. Surgical procedures, including endoscopic excision and dilation, tracheal/cricotracheal resection, and tracheostomy remain mainstay therapy, with adjuvant therapies including local and systemic steroid treatment, Mitomycin C, antibiotics, and anti-inflammatory medication.<sup>8-12</sup> Evidence for each of these medical therapies is inconsistent, demonstrating the need for rational new therapies to address scar tissue formation in the larynx and trachea.

Rapamycin (sirolimus, rapamine) is a macrocyclic antibiotic produced by the bacteria *streptomyces hygroscopicus* that functions as an immunosuppressive medication.<sup>13-15</sup> Rapamycin complexes with another protein to inhibit the mammalian target of rapamycin (mTor) and prevents activation of lymphocytes and other immune cells, thereby inhibiting the normal immune response.<sup>13,16,17</sup> It is an FDA-approved drug that is frequently used as

an antirejection agent for transplants as well as in coronary stents to reduce restenosis and accelerated arteriopathy.<sup>14,18,19</sup> There is also evidence to suggest that rapamycin is effective in reducing fibrosis and proliferation in a variety of different cell tissue types, including dermal, hepatic, and renal fibroblasts.<sup>20–22</sup>

The aim of this study was to determine whether rapamycin could inhibit human LTS fibroblast proliferation in vitro. Fibroblast proliferation is a crucial step for development of LTS, and collagen is usually implicated in deposition of scar tissue and in fibrosis. Therefore, we hypothesized that rapamycin would decrease LTS fibroblast proliferation and metabolism and reduce collagen production. We characterized the effect of rapamycin on fibroblasts through growth kinetics, morphology, gene expression, and cellular metabolism profile.

## Materials and Methods

### Laryngotracheal Fibroblast Isolation and Culture

Biopsy of human laryngotracheal stenosis was performed during routine suspension microlaryngoscopy, bronchoscopy, excision, and dilation of subglottic/tracheal stenosis in 5 patients. Informed consent was obtained from all patients to join this study, which was approved by the Johns Hopkins Institutional Review Board (NA\_00078310). Immediately after excision, tissue samples were placed in phosphate-buffered saline (PBS; Gibco Life Technologies by Invitrogen, Grand Island, New York, USA) supplemented with 5% penicillin/streptomycin (Gibco). Biopsy specimens were minced, and the tissue fragments were spread out evenly across uncoated plastic tissue culture flasks (BD Biosciences, San Jose, California, USA) and suspended in fibroblast growth medium at 37°C in 5% CO<sub>2</sub>-humidified atmosphere. Fibroblast growth medium consisted of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone by Thermo Scientific, Logan, Utah, USA), 100 U/mL penicillin (Gibco), 100 ug/mL streptomycin (Gibco), and 100× nonessential amino acids (NEAA; Gibco). Once cells were confluent (between 20–30 days), short-term 0.25% Trypsin-EDTA treatment (Gibco) proved effective in releasing only fibroblasts from the flasks, which were then plated into new flasks for expansion. Passage 2 or 3 of these primary fibroblast cell lines were used in the following experiments. Fibroblast presence was confirmed in culture with positive immunohistochemical staining of the anti-fibroblast antibody ER-TR7 (Santa Cruz Biotechnology, Inc, Dallas, Texas, USA) and negative staining for epithelial cells using the anti-human panCytokeratin antibody AF488 (Becton-Dickenson, Franklin Lakes, New Jersey, USA).

### Cell Proliferation by Cell Count and DNA Assay

After reaching confluence, the adherent LTS fibroblasts were trypsinized and passaged into 12-well culture plates (Falcon by BD Biosciences) containing fibroblast growth medium and seeded with 20,000 cells per well. Medium was aspirated and replaced daily with the following conditions: (1) control, normal fibroblast growth medium; (2) control-vehicle, medium containing vehicle solution, 1% dimethylsulfoxide (DMSO); (3) rapamycin-1, medium containing rapamycin (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in

DMSO at a concentration of 1ng/mL or  $10^{-10}$  M; (4) rapamycin-10, medium containing rapamycin dissolved in DMSO at a concentration of 10 ng/mL or  $10^{-9}$  M. These 2 concentrations were chosen due to their low and inhibitory doses in previous reports and order of magnitude difference between the doses.<sup>23,24</sup> All conditions were grown in triplicate. Cells were harvested on the sixth day of incubation. Prior to DNA assay, cell counts were performed on the aforementioned cells; twice on each well of trypsinized LTS fibroblasts. Then the LTS fibroblasts of each well were lysed (10 mM Tris, 1 mM EDTA, 0.1% Triton X-100 and 0.1 mg/ml Proteinase K), and DNA concentration was determined using the PicoGreen fluorescent method,<sup>25</sup> adapted from the manufacturer's protocol (Invitrogen Quant-iT PicoGreen dsDNA Reagent and Kits Protocol, Molecular Probes P11496). Each sample was run in duplicate for the assay. Fluorescence was read using the BioTek Synergy 2 Microplate Reader (BioTek Instruments, Winooski, Vermont, USA).

### Cell Staining and Histology

LTS fibroblasts were seeded in the 4 medium conditions on microscope cover glass slips (Fisher Scientific, Hampton, New Hampshire, USA) that were pretreated with 0.01% gelatin solution, dried, and sterilized. After 6 days, the LTS fibroblast glass cover slips were stained with Masson's Trichrome in order to visually assess differences in collagen deposition among the 4 medium conditions. The fibroblast glass cover slips were fixed with 10% formalin and stained following the manufacturer's instructions (Sigma-Aldrich #HT15 kit). Microscopic images of the stained sections were taken and analyzed with a Zeiss AX10 microscope (20× magnification, Carl Zeiss, Oberkochen, Germany).

### Gene Expression Analysis by Real-time Polymerase Chain Reaction

LTS fibroblasts were seeded in 6-well culture plates (Falcon by BD Biosciences) with 300,000 cells per well and grown in the 4 medium conditions in duplicate for 6 days. The RNeasy Micro Kit (Qiagen, Valencia, California, USA) was used for RNA extraction of each well, and the RNA was quantitated using the nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA). The iScript cDNA synthesis kit (BioRad, Hercules, California, USA) was used for cDNA synthesis according to the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed and monitored using the SYBR Green PCR Mastermix (Life Tech, Carlsbad, California, USA) on the StepOnePlus Real Time PCR System (Life Tech). Reaction mixtures were incubated for 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C, and finally 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C. For each sample, gene expression was corrected against Beta Actin level. All samples were run in duplicate. The level of expression of the target gene was calculated as  $2^{-Ct}$  as previously described.<sup>26</sup> The control LTS fibroblasts grown in normal growth medium were used for reference. Error was calculated by the standard deviation of  $Ct$ .<sup>27</sup> The fibrosis marker, collagen I (Col I), was assessed as previously described.<sup>28</sup>

### Cellular Metabolism Using Oxygen Consumption Rate Measurements

Bioenergetic analysis of oxygen consumption rate (OCR) provides a measurement of overall metabolic status of the cell.<sup>29</sup> OCR measurements of LTS fibroblasts were completed using a Seahorse XF24 Flux Analyzer (Seahorse Bioscience, Billerica, Massachusetts, USA) at 4

$\times 10^4$  cells per well. They were grown in 3 medium conditions for 2 days post seeding with respective medium changed daily. Due to constraints with the Seahorse experimental plate, only 3 conditions could be run simultaneously with sufficient replicates. The 3 medium conditions tested were the 2 control conditions and high-dose rapamycin. The assay used XF minimal assay medium supplemented with 25 mM glucose (Sigma), 1X GlutaMax (Invitrogen), and 1X NEAA (Invitrogen). Prior to the stress test, the cells were washed twice with this medium and incubated for 1 hour at 37°C in a humidified atmosphere without CO<sub>2</sub>. In order to evaluate the mitochondrial respiratory capacity, Oligomycin (an adenosine triphosphate [ATP] coupler), Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, an ATP uncoupler), Rotenone and Antimycin (mitochondrial inhibitors) at 10 uM, 4 uM, and 20 uM concentration, respectively, were injected into the medium at 34, 72, and 110 minutes, respectively. PicoGreen DNA quantization assay was performed on each well in order to normalize the OCR. DNA content was correlated with the total protein amount per well. Basal metabolic rate, ATP production, and maximal respiratory capacity were measured.

### Statistical Analysis

Results were expressed as averages  $\pm$  standard error of the mean. Statistical analysis was performed using the Wilcoxin rank-sum test. To minimize type I error, we performed a Bonferonni correction and calculated our corrected alpha level to be 0.05/3 groups = 0.017, with *P* values < .05 considered significant (STATA; StataCorp LP; College Station, Texas, USA).

## Results

### Rapamycin Decreases LTS Fibroblast Proliferation

Increased rapamycin dosing showed a decreased LTS fibroblast growth rate in all patients (Figures 1A, 2A) as well as in the mean (Figures 1B, 2B) for all patients. Fibroblasts proliferated in all medium conditions, with fibroblasts cultured in normal medium (mean cell count at day 6 =  $21.9 \times 10^4$ , SD =  $3.9 \times 10^4$ ) growing at a faster rate than fibroblasts cultured in DMSO (mean =  $17.9 \times 10^4$ , SD =  $5.0 \times 10^4$ ), low-dose rapamycin (mean =  $8.7 \times 10^4$ , SD =  $2.1 \times 10^4$ ) and high-dose rapamycin (mean =  $4.8 \times 10^4$ , SD =  $1.0 \times 10^4$ ). This represented a significant decrease in individual and average LTS fibroblast count cultured with high-dose rapamycin versus DMSO vehicle control (*P* = .0007) and normal (*P* = .0007) medium. Low-dose rapamycin also demonstrated a significant reduction in fibroblast count when compared to control (*P* = .0008) and normal (*P* = .0007) medium. There was also a significant decrease in DNA concentration of individual (Figure 1A) and average (Figure 1B) LTS fibroblasts cultured with high-dose rapamycin versus DMSO vehicle control (*P* = .0007) and normal (*P* = .0007) medium. Furthermore, there was a significant reduction in DNA concentration for fibroblasts grown in low-dose rapamycin versus normal (*P* = .0026) and vehicle medium (*P* = .0015). The DNA per cell was not significantly different in fibroblasts cultured in different cell medium types.

### Rapamycin-treated Fibroblasts Demonstrate Reduced Collagen Staining

LTS fibroblasts were stained with Masson's Trichrome in order to visualize collagen deposition, which stains blue. Histological evaluation of LTS cultured cells after treatment of rapamycin is shown in Figure 3.

### Rapamycin-treated Fibroblasts Have Reduced Collagen I Expression

RT-PCR demonstrated decreased Col I gene expression in rapamycin-treated LTS fibroblasts compared to control LTS fibroblasts in all 5 patients (Figure 4A). The average of all 5 patients also showed a linear trend in which increasing rapamycin concentration resulted in a decrease in Col I gene expression (Figure 4B). There was a significant decrease in Col I expression between LTS fibroblasts grown in high-dose 10 ng/mL rapamycin and in control fibroblast medium ( $P = .0054$ ) and in DMSO vehicle medium ( $P = .0102$ ). There was a significant decrease in Col I expression in LTS fibroblasts grown in low-dose rapamycin versus normal medium ( $P = .0074$ ), though not when compared with the vehicle medium ( $P = .0784$ ) control.

### Rapamycin Decreases the Metabolic Rate of LTS Fibroblasts

LTS patient-derived fibroblasts were less metabolically active than those grown in normal and vehicle medium controls (Figures 5, 6). When averaged together, there were significant decreases in basal respiration (Figure 5A), ATP production (Figure 5B), and maximal respiration (Figure 5C) between LTS fibroblasts treated with high-dose rapamycin and those cultured in DMSO control and fibroblast growth medium. Maximal respiration decreased to 68.6 pMoles of oxygen/min/10 mg of protein units from 96.9 pMoles for DMSO medium ( $P = .0002$ ) and from 97.0 pMoles for the normal medium control ( $P = .0022$ ). Similarly, ATP production decreased to 66.8 pMoles of oxygen/min/10 mg of protein units from 88.1 pMoles for the DMSO control ( $P = .0006$ ) and from 83.3 for the normal medium control ( $P = .0003$ ). Lastly, basal respiration decreased to 78.6 pMoles of oxygen/min/10 mg of protein units from 108 pMoles for DMSO medium ( $P = .0002$ ) and from 101 pMoles in the normal medium control ( $P = .0014$ ). Figure 6 demonstrates the difference in a representative patient's fibroblast oxygen consumption rate between the 3 medium conditions: normal medium control, DMSO control, and high-dose rapamycin. High-dose rapamycin decreased the basal metabolic rate (difference in peak OCR prior to Oligomycin and lowest OCR post Rotenone/Antimycin) and ATP production (difference in OCR prior to and post Oligomycin injection) and decreased maximal respiratory capacity (difference between peak OCR post FCCP injection and the nonmitochondrial respiration) in LTS fibroblasts.

## Discussion

The results of this in vitro study suggest rapamycin as a potential anti-fibroblast therapy for LTS. As shown, we observed a decrease in LTS fibroblast proliferation, function, and metabolism with rapamycin. High-dose rapamycin demonstrated a significant reduction in every outcome measured as compared to vehicle medium and normal medium controls in the cells from the 5 patients included in this study. While the 10 ng/mL rapamycin concentration represented the "high dose" in this study, it is relatively low compared to other reports.<sup>23,24</sup> Doses as high as 10 ug/mL inhibit fibroblast proliferation with minimal



toxicity.<sup>23</sup> The results in these 5 LTS patients are supported by rapamycin's effect on fibroblasts in other organ systems, including the lung, liver, and skin, by decreasing expression and deposition of collagen type I and reducing cell proliferation.<sup>21,22,24</sup> This study suggests a potential mechanism by assessing cellular respiration in LTS fibroblasts, which showed a reduced mitochondrial metabolic rate after treatment with rapamycin.

Bioenergetic analysis is a novel technique that monitors the oxygen consumption and extracellular acidification at a cellular level.<sup>30-32</sup> The key parameters of basal respiration, ATP production, and respiratory capacity may be measured to assess mitochondrial metabolism.<sup>30,32</sup> Importantly, maximal respiratory capacity was significantly reduced in rapamycin-treated fibroblasts when nonmitochondrial respiration levels were normalized between control and rapamycin-treated groups. As seen in this study, the decreased basal respiration, decreased ATP production, and maximal respiratory capacity after treatment suggest that rapamycin interferes with mitochondrial energy production, resulting in decreased fibroblast function and collagen proliferation.

In clinical application for the treatment of LTS, we expect rapamycin to exert an immunosuppressive effect in addition to antiproliferative effects. Rapamycin has FDA approval for immunosuppression to prevent rejection in renal transplant patients and is used to coat coronary stents to reduce inflammation and collagen synthesis.<sup>15,33</sup> Furthermore, rapamycin was shown to disrupt mTOR signaling in fibroblasts and macrophages and reduce kidney fibrosis.<sup>20</sup> Therefore, we are encouraged by the potential of rapamycin treatment as adjuvant therapy for LTS. Modulating the immune response may be more effective than current medical therapies. In topical application, rapamycin could assist in suppressing the inflammatory response and fibroblast proliferation and collagen synthesis seen in LTS while avoiding side effects seen with systemic therapy. Ultimately, a rapamycin-eluting stent could direct sustained local immunotherapy and anti-fibroblast effect at the area of fibrosis while providing mechanical forces on the tracheal wall. Further *in vivo* investigation is required to support our data and hypothesis.

While this study demonstrates the benefits of rapamycin on human LTS cells, there are several limitations. It is an *in vitro* study of human cells, and the behavior of the LTS fibroblasts on culture dishes may not be representative of cells *in vivo*. While only passage 2 and 3 cells were used in experiments, the repeated passaging and freeze-thawing may have an effect on cell phenotype. Lastly, it is important to note that there was variability among LTS fibroblasts in the 5 different patients (Figures 1A, 2A, 4A, and 6) with varying response to rapamycin appreciated. This patient-specific variability in therapeutic response may be encountered with any clinical treatment and may be due to the heterogeneity of the disease process or possibly genetic differences in wound healing. We are encouraged by the significant anti-fibroblast effect of rapamycin when the results from the 5 patients with LTS were averaged.

In summary, rapamycin demonstrated an anti-fibroblast effect by reducing the proliferation, metabolism, and collagen deposition of human LTS fibroblast *in vitro*. The bioenergetic effects of rapamycin demonstrated a significant decrease in oxidative phosphorylation of LTS fibroblasts, suggesting a potential mechanism for the reduced proliferation and

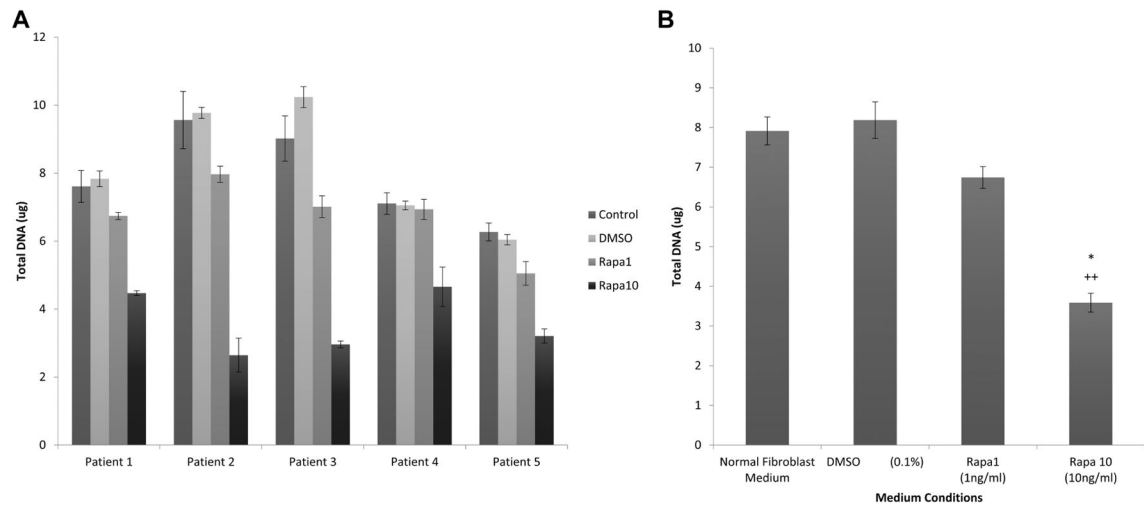
differentiation. Rapamycin's anti-fibroblast effects combined with its immunosuppressive action suggest a promising adjuvant therapy for the treatment of laryngotracheal stenosis. Further studies are underway to compare the metabolism of normal and scar fibroblasts isolated from patients with LTS as well as to assess rapamycin's effect on laryngotracheal stenosis in an in vivo model.

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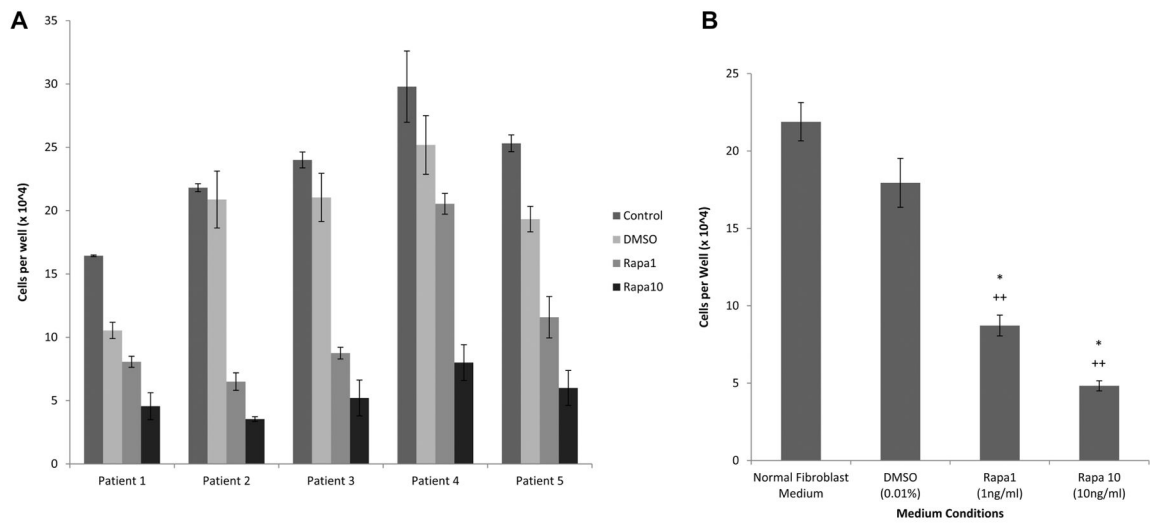


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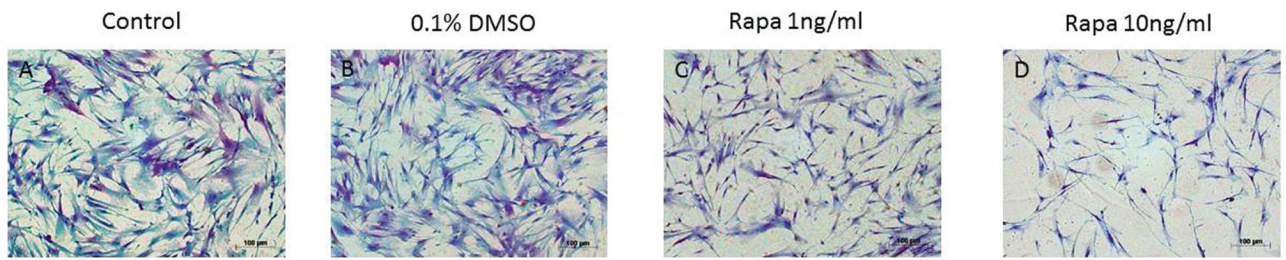
**Figure 1.**

Rapamycin inhibits fibroblast proliferation. Rapamycin significantly reduced DNA in laryngotracheal stenosis (LTS)–derived fibroblasts (A) compared to controls. When averaged (B), DNA was reduced for low- and high-dose rapamycin versus normal ( $P = .0026$ ,  $P = .0007$ ) and dimethylsulfoxide (DMSO) vehicle ( $P = .0015$ ,  $P = .0007$ ) controls.

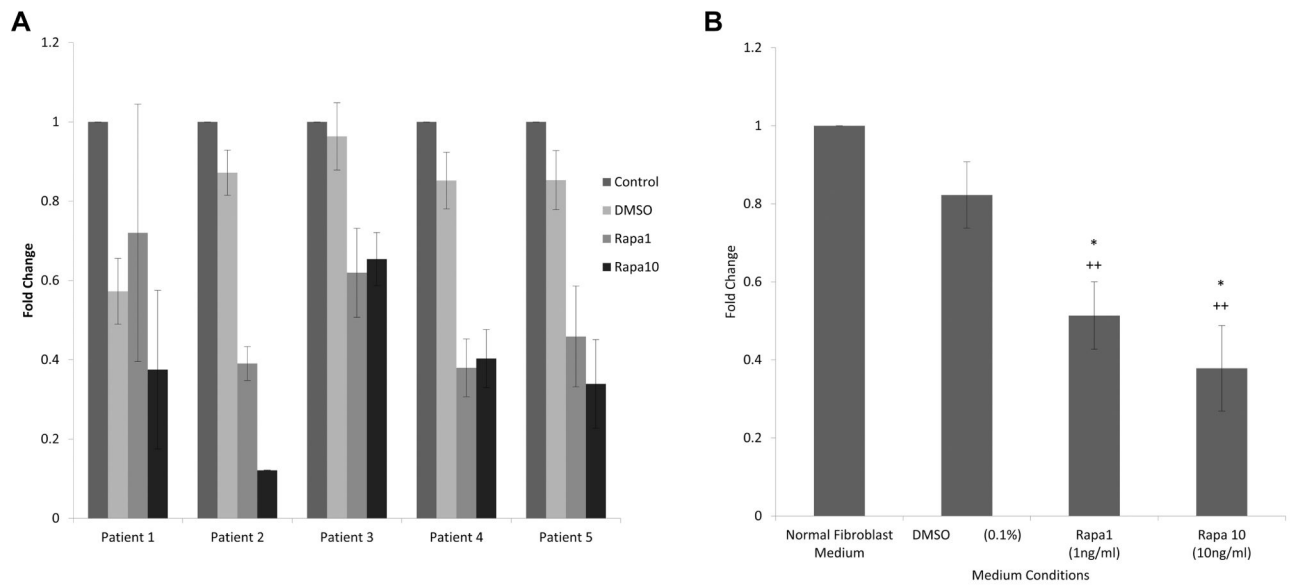


**Figure 2.**

Rapamycin reduces fibroblast count. Fibroblasts were significantly lower after rapamycin treatment (A) compared with controls. Average cell count (B) was reduced for low- and high-dose rapamycin versus normal ( $P = .0001$ ,  $P = .0001$ ) and dimethylsulfoxide (DMSO) ( $P = .0001$ ,  $P = .0001$ ) controls.

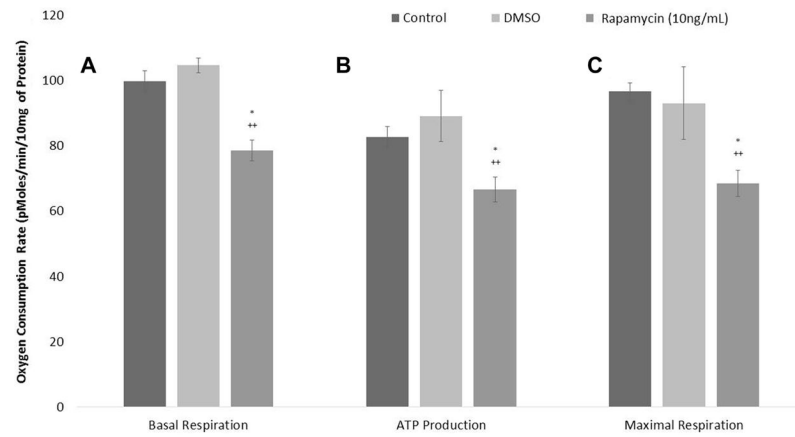


**Figure 3.** Fibroblast morphology. (A) Normal and (B) dimethylsulfoxide (DMSO) vehicle controls demonstrate confluent fibroblasts with intense blue staining for collagen compared with fibroblast proliferation and collagen deposition after treatment with (C) low- and (D) high-dose rapamycin.



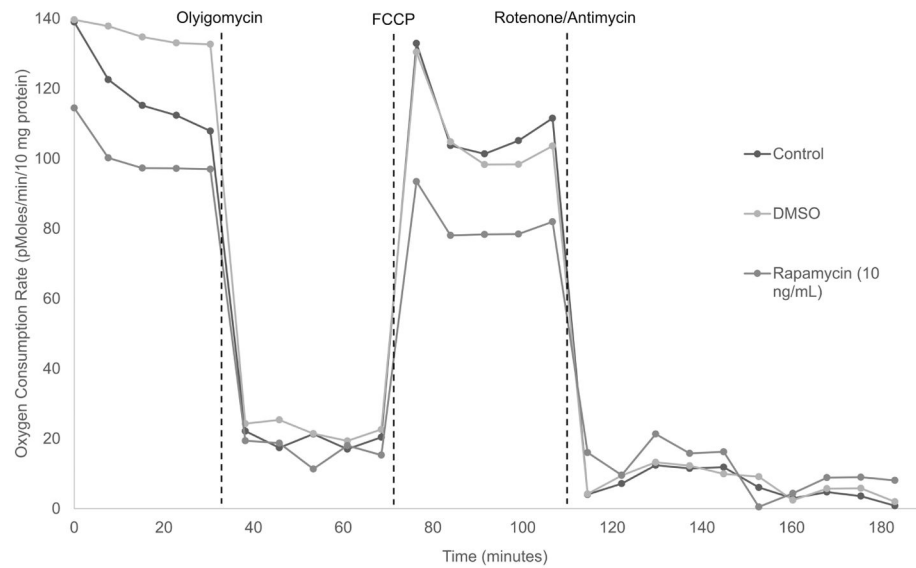
**Figure 4.**

Rapamycin inhibits collagen 1 expression. (A) Four patients had significantly reduced collagen 1 expression after rapamycin treatment. When averaged (B), high-dose rapamycin reduced collagen 1 expression compared to normal ( $P = .0054$ ) and dimethylsulfoxide (DMSO) ( $P = .0102$ ) controls.



**Figure 5.** Rapamycin decreases the metabolic rate of laryngotracheal stenosis (LTS) fibroblasts. (A) Mean basal respiration, (B) adenosine triphosphate (ATP) production, and (C) maximal respiration significantly decreased with high-dose rapamycin when compared to normal medium and vehicle medium controls.





**Figure 6.**

Oxygen consumption rate in representative patient-derived fibroblasts. LTS-derived fibroblasts were seeded in FGM, FGM + DMSO, and FGM + DMSO + rapamycin (10 ng/mL) medium. ATP production, basal, and FCCP-coupled OCR were significantly reduced with rapamycin treatment. ATP, adenosine triphosphate; DMSO, dimethylsulfoxide; FCCP, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; FGM, fibroblast growth medium; OCR, oxygen consumption rate.