

Perspective

Rapamycin in fibrotic diseases: beneficial or detrimental agent?

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Rapamycin was first isolated from a strain of *Streptomyces hygroscopicus* in the early 1970s from a soil sample taken on Easter Island.¹ Because the antiproliferative effects of rapamycin on yeast cell, as well as B and T lymphocytes, it was first identified as an antimicrobial agent with potent immunosuppressive activity and has been used in antirejection therapy.^{2,3}

The discovery of rapamycin led to the identification and clone of the mammalian target of rapamycin (mTOR). mTOR is a highly conserved serine-threonine kinase shown to integrate and coordinate diverse signaling pathway mediated by growth factors, nutrient availability and energy status to play a critical role in cell growth, proliferation, division, migration, and survival.⁴ Structurally, mTOR is a part of two signaling complexes, mTOR complex 1 (mTORC1) and mTORC2. Rapamycin is a potent, highly specific inhibitor of mTORC1 and does not attenuate any kinase other than mTOR.⁴ After diffusing into cells, rapamycin forms a complex with FK506-binding protein 12 (FKBP-12) and then binds and inhibits mTOR phosphorylation and inactivates ribosomal S6 kinase 1 (S6K1) and the eukaryotic initiation factor 4E (eIF4E) inhibitory binding protein (4E-BP).⁴ Via inhibiting the mTOR signal axis, rapamycin modulates malignant cellular growth, progression of fibrotic disorders. The current review will give a brief overview on the rapamycin/mTOR network relevant to fibrotic diseases and will mainly focus on recent scientific debates on rapamycin's regulatory role in fibrosis models both *in vitro* and *in vivo* (Table 1).

RAPAMYCIN INHIBITS TYPE I COLLAGEN, FIBRONECTIN, α -SMA, TIMP-1 AND AUGMENT MMP-1/9 EXPRESSION: *IN VITRO* EVIDENCES FOR ANTIFIBROTIC EFFECTS

Dysregulated wound healing response to a variety of acute and/or chronic stimuli contributes to fibrotic disorders. Fibrosis will ultimately occur due to an over production of extracellular matrix components (Figure 1), including type I collagen.⁵ These fibrillar collagen synthesis and deposition will disrupt normal organ architecture, induce abnormal tissue remodeling and organ malfunction. (Myo)fibroblasts are the main effector cells for matrix production (Figure 1). They recruited from a variety of sources such as resident mesenchymal cells, bone marrow progenitors (circulating fibrocytes) and epithelial or endothelial cells via a process called

epithelial or endothelial mesenchymal transition (EMT or EndMT).⁶

In addition to the increase in collagen production, the accumulation of extracellular matrix (ECM) is also due to an insufficient matrix turnover (Figure 1). Physiologically, the metabolism of ECM proteins is delicately regulated by proteases and their corresponding inhibitors, including the matrix metalloproteases (MMPs)/ tissue inhibitors of matrix metalloproteinases (TIMPs) and plasminogen, plasminogen activators (PAs)/ plasminogen activator inhibitors (PAIs).⁷ Pathologically, if the balance becomes in favor of TIMPs/PAIs, ECM resolution will be difficult and may cause insufficient fibrillar matrix destruction and fibrotic lesions.⁸ So agents targeting to modulate ECM production and degradation would have potential benefits for the treatment of fibrotic diseases.

Poulalhon et al⁹ found that rapamycin attenuated expression of both types I and III collagens in human lung fibroblast (WI-26), while significantly enhanced the expression of interstitial collagenase (MMP-1) at the protein and mRNA levels. Another study found that rapamycin significantly increased intracellular fibronectin expression in rabbit lens epithelium cells (rLECs).¹⁰ Simultaneously, it was found that α -SMA expression of NIH3T3 cells was remarkably reduced by rapamycin treatment.¹¹ In line with these results, Shegogue and Trojanowska¹² also showed inhibition of mTOR activity using rapamycin remarkably reduced collagen mRNA levels of human fibroblast *in vitro*. These results drew the conclusion that rapamycin had direct antifibrotic activities *in vitro* through modulating ECM synthesis and

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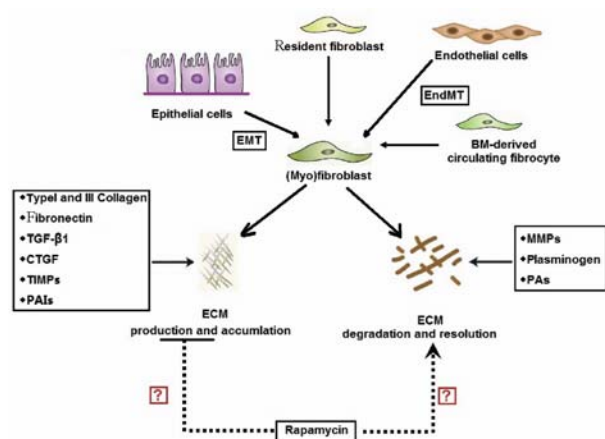


Figure 1. ECM excessive production as well as insufficient turnover are the hallmarks of fibrotic diseases. (Myo)fibroblasts are the key effector cells for ECM accumulation. They derived from various progenitors, such as resident mesenchymal cells, bone marrow derived circulating fibrocytes and epithelial or endothelial cells via a process called EMT or EndMT. Type I collagen, together with type III collagen and fibronectin constitutes the main components of ECM. TGF- β 1 and CTGF are the master profibrotic cytokines for (myo)fibroblast to synthesis ECM proteins. ECM turnover is balanced by proteases and their corresponding inhibitors, such as MMPs/ TIMPs and plasminogen, PAs/ PAIs. Rapamycin could modulate these pro- or anti-fibrotic gene expressions to exert its distinct function in fibrotic disorders.

turnover *in vitro* and may provide a suitable therapy method for fibrogenic diseases (Figure 2A).

RAPAMYCIN ATTENUATES LUNG, KIDNEY, HEPATIC AND OTHER TISSUE FIBROSIS THROUGH DIFFERENT MECHANISMS: *IN VIVO* EVIDENCES FOR ANTIFIBROTIC EFFECTS

A series of animal or human models were used *in vivo* to examine the pathogenesis and treatment of fibrotic disorders, including bleomycin induced mouse models of Systemic Sclerosis,¹³ transforming growth factor- α induced pulmonary fibrosis,¹⁴ bile duct ligation induced hepatic fibrosis¹⁵ and chronic allograft nephropathy (CAN).¹⁶ In their remarkable work, Yoshizaki et al¹³ showed that rapamycin treatment reduced skin and lung fibrosis of bleomycin-induced SSc model mice. They suggested that attenuated production of fibrogenic cytokines, including interleukin-4 (IL-4), IL-6, IL-17, and transforming growth factor (TGF)- β 1 was responsible for the beneficial effects of rapamycin. Consistent with these data, Korfhagen et al¹⁴ found that rapamycin prevented the onset of fibrosis and blocked further development in established pulmonary fibrosis in cytokine-induced lung fibrosis model. The accumulation of total lung collagen, weight loss, and deterioration in pulmonary mechanics were all attenuated. Simultaneously, Bridle et al¹⁵ showed that rapamycin inhibited hepatic fibrosis of rat model via inhibiting procollagen type I, α -smooth muscle actin (SMA) production, attenuating epithelial-mesenchymal transition and hepatic stellate cell proliferation. Pontrelli et al¹⁶ also showed that rapamycin reduced

glomerulosclerotic lesions in renal biopsy specimens through reducing PAI-1 expression. All of these important findings above provide *in vivo* evidence for the antifibrotic activity of rapamycin in different animal models of fibrosis via attenuating multiple profibrogenic pathways (Figure 2B).

Studies on the modulatory effects of rapamycin in other organs *in vivo* seem rather insufficient. Some case reports showed that rapamycin may be effective on the treatment for chronic intestinal disease and scleroderma.¹⁷⁻¹⁹

RAPAMYCIN AUGMENTS CTGF, TIMP-1, PAI-1 AND INHIBIT MMP-1/9 EXPRESSION: *IN VITRO* EVIDENCES AGAINST ANTIFIBROTIC EFFECTS

The antifibrotic effects hypothesis of rapamycin was challenged by some other *in vitro* studies which showed that rapamycin has a profibrotic property. In their significant studies, Osman et al²⁰ demonstrated that rapamycin treatment caused an increase in profibrotic gene expression as exemplified by the connective tissue growth factor (CTGF) and PAI-1 in rat mesangial cells (MC). These profibrotic effects were exerted by activation of the TGF β 1/Smad signaling cascade. CTGF plays an important role in fibrogenic responses via promoting fibroblasts proliferation and extracellular matrix synthesis.²¹ Concomitantly, Osman et al²² also found that rapamycin promoted cytokine-triggered TIMP-1 expression in rat MC. On the contrary, the expression of MMP-9 was remarkably reduced. The differential modulation on protease/antiprotease system indicates that rapamycin may be a potent drug to promote fibrotic process. In another study, Ma and fellows²³ showed that incubation of human umbilical vein endothelial cells (HUVECs) with rapamycin strongly reduced the expression of t-PA. However, the expression of PAI-1 was significantly induced. Taken together, these data imply that rapamycin may have profibrotic activities via various mechanisms *in vitro* (Figure 2C).

RAPAMYCIN DOES NOT ATTENUATE PROGRESSION OF LUNG, LIVER AND KIDNEY FIBROSIS: *IN VIVO* EVIDENCES AGAINST ANTIFIBROTIC EFFECTS

Contrary to these studies arguing for antifibrotic activity of rapamycin *in vivo*, Madala et al²⁴ showed that rapamycin increased weight loss and decreased survival of bleomycin-treated *Sf tpc+/+* and *Sf tpc-/-* mice. Furthermore, rapamycin did not reduce the fibrotic disease in the prophylactic or rescue experiments and even augmented airway resistance and reduced lung compliance. Rapamycin also caused a significantly increased expression of pro-fibrotic Th2 cytokines and reduced expression of INF- γ . All of these results showed that rapamycin treatment could not attenuate the onset or progression of lung fibrosis, rather it may promote the fibrotic process through different ways. In addition, Renken

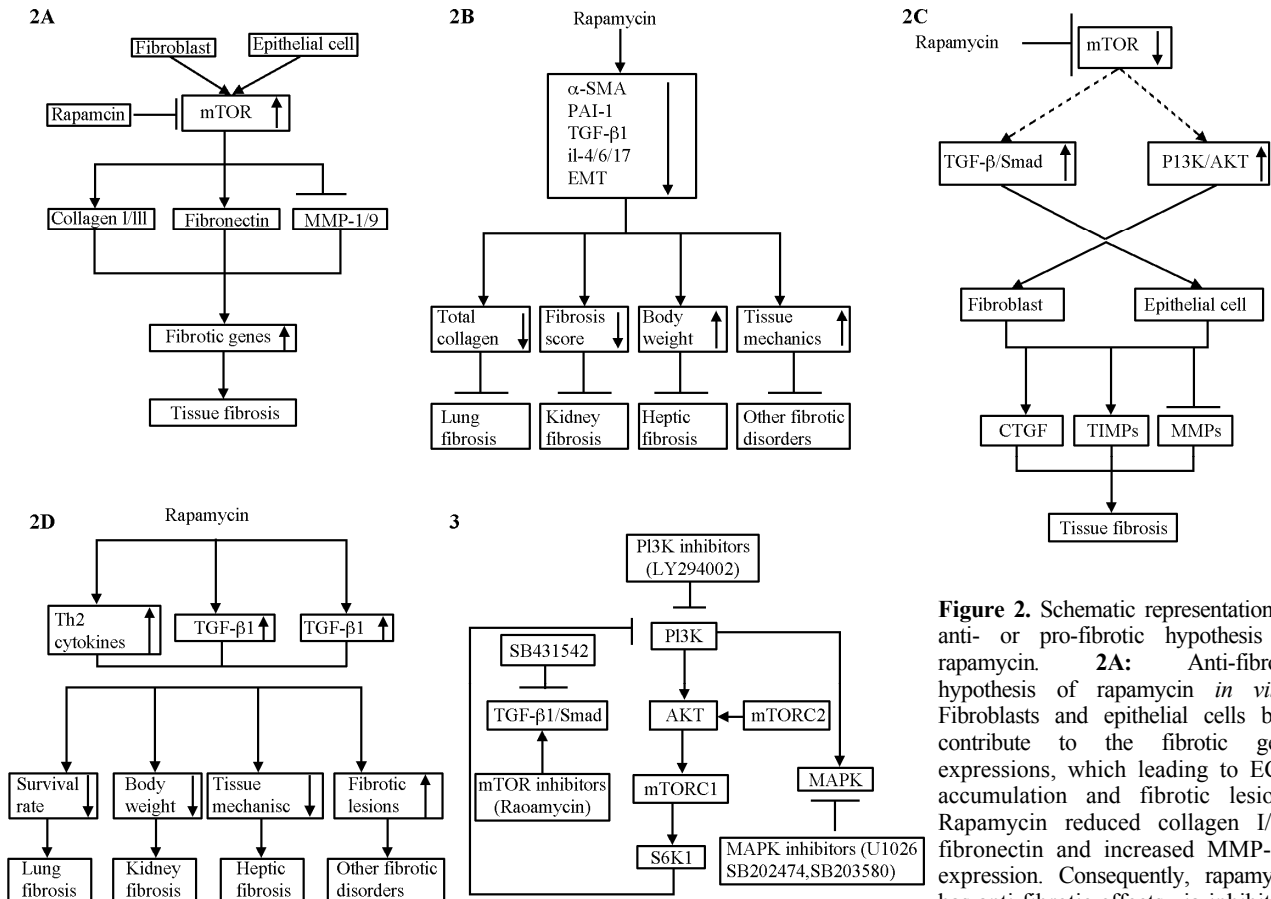


Figure 2. Schematic representation of anti- or pro-fibrotic hypothesis of rapamycin. **2A:** Anti-fibrotic hypothesis of rapamycin *in vitro*. Fibroblasts and epithelial cells both contribute to the fibrotic gene expressions, which leading to ECM accumulation and fibrotic lesions. Rapamycin reduced collagen I/III, fibronectin and increased MMP-1/9 expression. Consequently, rapamycin has anti-fibrotic effects via inhibiting

the major ECM accumulation *in vitro*. **2B:** Anti-fibrotic hypothesis of rapamycin *in vivo*. Rapamycin decreased total collagen deposition, fibrosis score and increased body weights and organ functions. Rapamycin exerted its modulatory effects via multiple pathways, including inhibiting expressions of fibrotic cytokines, promoting ECM resolution, and etc. **2C:** Pro-fibrotic hypothesis of rapamycin *in vitro*. Inhibition of the mTOR activity with rapamycin results in a hyperactive receptor tyrosine kinase (RTK)/PI3K and TGF-β/Smad pathway. These secondarily activated pathways may contribute to the accumulation of profibrotic genes produced by fibroblasts and/or epithelial cells. Finally, rapamycin showed pro-fibrotic *in vitro*. **2D:** Pro-fibrotic hypothesis of rapamycin *in vivo*. Rapamycin may upregulated Th2 cytokines, TGF-β1, PAI-1 *in vivo*. These pro-fibrotic genes may counteract other beneficial effects of rapamycin and result in a pro-fibrotic function.

Figure 3. mTOR related signal pathways. mTOR is a part of two distinct complexes, mTORC1 and mTORC2. mTORC1 phosphorylates and activates S6K1 resulting in the initiation of transcription and translation. mTORC2 phosphorylates and activates Akt. Rapamycin is a specific inhibitor of mTORC1 and does not affect mTORC2's function. Emerging evidence has shown that inhibiting mTOR would promote a negative feedback loop which results in the activation of proliferative signals such as Akt or MAPK that in turn counteract the inhibitory properties of rapamycin. Rapamycin also induces TGF-β1/Smad cascade in some cell type. These different signal pathways activated due to inhibition of mTOR may involve in the profibrotic effects of mTOR inhibitors. Combination treatment with inhibitors of PI3K-AKT (Wortmannin), MAPK (U1026, SB202474, SB203580), TGF-β1-Smad (SB431542) cascade may augment the antifibrotic activity of rapamycin.

et al²⁵ also showed that rapamycin treatment failed to attenuate progression of kidney and liver fibrosis in PCK rats. In line with these data, many other studies^{26,27} showed that rapamycin induced TGF-β1 production *in vivo*. Also, some recent case reports showed that rapamycin would induce interstitial pneumonia and even fibrotic lesions when used in renal transplant or renal cell carcinoma patients.^{28,29} Collectively, these data showed that rapamycin has profibrotic abilities *in vitro* (Figure 2D).

IN VITRO AND IN VIVO EVIDENCES FOR AND AGAINST ANTIFIBROTIC ACTIVITY OF RAPAMYCIN: HOW TO RECONCILE?

As is mentioned above, we have listed a lot of evidences

for or against rapamycin's antifibrotic effects *in vitro* and *in vivo*. We seem rather confused with the real modulation effects of rapamycin on fibrotic disorders because all the researches with the opposite data seem reasonable. So it's a high time to debate now. Issues discussed below may serve to reconcile the discrepancy: (1) Various doses of rapamycin and different animal models. Lower or higher doses of rapamycin with different length of treatment time would be involved in the paradox about rapamycin's modulatory effects on fibrotic diseases. Studies with lower dose (such as 2.0 mg/kg, or 2.5 mg/kg) of rapamycin administration showed a significant antifibrotic effects of this agent in bleomycin^{30,31} induced lung fibrosis. However, larger dose of rapamycin (such as 4 mg/kg) would exert no antifibrotic or even profibrotic effects in bleomycin-

Table 1. Studies of pro- or anti-fibrotic effects of rapamycin on fibrotic disorders *in vitro* and *in vivo*

Experimental manipulation	Statistical methods	Effects of rapamycin	Conclusions	References
Human lung fibroblasts (WI-26) culture	Not mentioned	Collagen I/III ↓ MMP-1↑	Anti-fibrotic effects	Poullahon N, et al. ⁹
Rabbit lens epithelium cells (rLECs) culture	Two-tailed Student's <i>t</i> -test	Migration↓ Fibronectin↓	Anti-fibrotic effects	Liu H, et al. ¹⁰
NIH3T3 cells culture	One-way ANOVA followed up with Tukey's test or <i>t</i> -test	pS6K↓ α-SMA↓	Anti-fibrotic effects	Chen G, et al. ¹¹
Human fibroblasts (from foreskins of healthy newborns) culture	Student's <i>t</i> -test	Col1A1↓ Col1A2↓	Anti-fibrotic effects	Shegogue D, et al. ¹²
Tight skin (TSK/+) mice and bleomycin induced SSc model mice	Mann-Whitney <i>U</i> test	IL-4/6/17, TGF-β1↓ Hypodermal thickness↓ Hydroxyproline conten↓	Anti-fibrotic effects	Yoshizaki A, et al. ¹³
Lung-specific TGF-α expression transgenic mouse model of lung fibrosis	One-way ANOVA	Collagen content ↓ Body weights ↑ Lung function ↑	Anti-fibrotic effects	Korfhagen TR, et al. ¹⁴
Bile duct ligation induced rats' hepatic fibrosis model	Student's <i>t</i> -test	Procollagen ↓ α-SMA↓ EMT marker ↓ Histopathological Scoring ↓	Anti-fibrotic effects	Bridle KR, et al. ¹⁵
Chronic allograft nephropathy (CAN) model	Student's <i>t</i> test Chi-square test	PAI-1 ↓ Mean total glomerular area ↑	Anti-fibrotic effects	Pontrelli P, et al. ¹⁶
Bleomycin induced rats lung fibrosis model	Unpaired <i>t</i> -test Mann-Whitney U-test	Lung-collagen accumulation↑ lung weight↓	Anti-fibrotic effects	Simler NR, et al. ³⁰
Bleomycin induced C57Bl/6 mice lung fibrosis model	Mann-Whitney test Kruskal-Wallis test	fibrocyte infiltration ↓ lung collagen deposition↓	Anti-fibrotic effects	Mehrad B, et al. ³¹
Rat mesangial cells (MC) culture	Student's <i>t</i> test ANOVA test	PAI-1 ↑ CTGF ↑	Pro-fibrotic effects	Osman B, et al. ²⁰
Rat mesangial cells (MC) culture	Student's <i>t</i> test ANOVA test	TIMP-1↑ MMP-9↓	Pro-fibrotic effects	Osman B, et al. ²²
Umbilical vein endothelial cells (HUVECs) culture	Student's <i>t</i> test ANOVA test	PAI-1↑ tPA↓	Pro-fibrotic effects	Ma Q, et al. ²³
Bleomycin induced Sf tpc ^{-/-} mice lung fibrosis model	One-way ANOVA	Weight loss ↑ Survival rate↓ Airway resistance↑ Th2 cytokines↑ INF-γ↓	Pro-fibrotic effects	Madala SK, et al. ²⁴
Weaned PCK rats model of human ARPKD	Student's <i>t</i> -test Mann-Whitney rank test	mTOR signaling: undetectable Fibrosis and cyst area, renal function: unimproved	Pro-fibrotic effects	Renken C, et al. ²⁵

induced lung fibrosis of SP-C-deficient mice model.²⁴ In a cytokine (TGF-α) induced lung fibrosis model, even large dose of rapamycin (4 mg/kg) could prevent fibrosis as well as prevent progression of fibrosis when rapamycin was administered after extensive fibrosis had already developed.¹⁴ So varying doses of rapamycin and animal models should be concerned in the different remarkable data. (2) Intrinsic or acquired 'rapamycin resistance' may serve to explain rapamycin's failure to exhibit antifibrotic effects.^{25,32-34} Rapamycin is a specific but not a complete inhibitor of mTORC1. S6K activity has been shown to be completely inhibited by rapamycin, however, the phosphorylation of 4E-BP1 is not affected by it.³⁵ Therefore after rapamycin treatment, some mTOR activities can still be executed by activated eIF-4E. Any rapamycin resistant activity of mTORC1 would induce to a more complex regulatory process in fibrotic disorders after rapamycin treatment. However, whether this "resistance" phenomenon really exists in fibrotic disorders should be further elucidated. (3) The complexity and widely crosslink between mTOR system and other signaling pathways activated secondarily to the mTOR inhibition by rapamycin. It was already known that there exist negative feedback loops in

mTOR/S6K1/4EBP1 signal system.³⁶⁻³⁸ Activation of mTOR would lead to phosphatidyl inositol 3kinase (PI3K) and mitogen-activated protein kinase (MAPK) inhibition through the negative feedback loop stemming from S6K1. While inhibition mTOR activity with rapamycin and its analogue results in a hyperactive receptor tyrosine kinase (RTK) / (PI3K) pathway, increasing the signal toward the Ras-Raf1-MEK1/2- (extracellular signal-regulated kinase) ERK pathway.³⁶⁻³⁸ In addition, rapamycin administration also could induce TGF-β1/Smad signaling cascade.²⁰ Beyond this, additional interactions between mTORC1 (target of rapamycin) and mTORC2-mediated signalling are likely to exist. So alternative activation of signal pathways induced by mTOR inhibition may contribute to profibrotic effects of rapamycin both *in vitro* and *in vivo* and combined inhibition of PI3K-AKT/MAPK/TGF-β1 and mTOR signal pathways may have additive anti-anfibrotic effects (Figure 3).

CONCLUSION

In this review, we listed evidences for and against rapamycin's effects on fibrotic disorders and aimed to explain the remarkable debates. We believe rapamycin

has a more sophisticated modulatory effect on fibrotic diseases *in vitro* or *in vivo* than we used to think. The real or final effects of rapamycin depend on the balance of changes in various pro- or anti-fibrotic gene expressions as well as on the synergy of mTOR signal cascade and other signal pathways activated by mTOR inhibition. Actually, rapamycin is “a coin of two sides”, how to amplify anti-fibrogenic and avoid pro-fibrotic effects is a great challenge for investigators. Careful consideration and evaluation of *in vitro* or *in vivo* studies can promote the possibility of identifying the effects and safeness of this agent.

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