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TOPIC HIGHLIGHT

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Transforming growth factor-β and fibrosis

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Abstract

Transforming growth factor-β (TGF-β), a prototype of multifunctional cytokine, is a key regulator of extracellular matrix (ECM) assembly and remodeling. Specifically, TGF-β isoforms have the ability to induce the expression of ECM proteins in mesenchymal cells, and to stimulate the production of protease inhibitors that prevent enzymatic breakdown of the ECM. Elevated TGF-β expression in affected organs, and subsequent deregulation of TGF-β functions, correlates with the abnormal connective tissue deposition observed during the onset of fibrotic diseases. During the last few years, tremendous progress has been made in the understanding of the molecular aspects of intracellular signaling downstream of the TGF-β receptors. In particular, Smad proteins, TGF-β receptor kinase substrates that translocate into the cell nucleus to act as transcription factors, have been studied extensively. The role of Smad3 in the transcriptional regulation of type I collagen gene expression and in the development of fibrosis, demonstrated both *in vitro* and in animal models with a targeted deletion of *Smad3*, is of critical importance because it may lead to novel therapeutic strategies against these diseases. This review focuses on the mechanisms underlying Smad modulation of fibrillar collagen expression and how it relates to fibrotic processes.

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Key words: Collagen; Connective tissue growth factor; Fibrosis; Smad; Transforming growth factor-β

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INTRODUCTION

Fibrosis is a complex tissue disease whose predominant characteristics are the excessive and abnormal deposition of extracellular matrix (ECM) components[1,2], that may affect various organs, including lung, liver, kidney and skin. From a clinical point of view, fibrosis may be considered as a somewhat irreversible state of scar tissue, during which resolution of the healing process does not occur. Long-term activation of fibroblasts in the affected organs results in massive fibrous ECM deposition and excessive fibroblast/myofibroblast proliferation, thus contrasting with normal wound healing during which feedback mechanisms counterbalance the initial fibroblast activation into myofibroblasts[3].

Much attention is focused on the role of many cytokines and growth factors, a group of diverse molecules derived from blood cells such as platelets, or elaborated locally by mesenchymal and epithelial cells, that contribute to the fibrogenic process[1,4]. Among them, the profibrotic proteins transforming factor-β (TGF-β) and connective tissue growth factor (CTGF) are considered master switches for the induction of the fibrotic program. TGF-β induces fibroblasts to synthesize and contract ECM[5,6], and CTGF, induced by TGF-β, is considered as a critical downstream mediator of TGF-β effects on fibroblasts[7,8]. In this overview, we will discuss the progress made in understanding the central role of TGF-β in fibrotic diseases.

TGF-β AND RECEPTORS ACTIVATION

TGF-β activation

More than 60 TGF-β family members have been identified in multicellular organisms. Among these, there are three TGF-βs, five activins and at least eight Bone Morphogenetic Proteins (BMPs), all encoded by distinct genes (Figure 1)[9]. The three mammalian TGF-β isoforms, TGF-β1, 2, and 3 are secreted as latent precursor molecules (LTGF-β) that contain an amino-terminal hydrophobic signal peptide region, the latency associated peptide (LAP) region and the C-terminal potentially bioactive region[10]. The LTGF-β is usually complexed with latent TGF-β-binding proteins (LTBP), requiring activation into a mature form for receptor binding and subsequent activation of signal transduction pathways. The LTBP is removed extracellularly by either proteolic cleavage by various proteases such as plasmin, thrombin, plasma transglutaminase, or endoglycosylases, or by

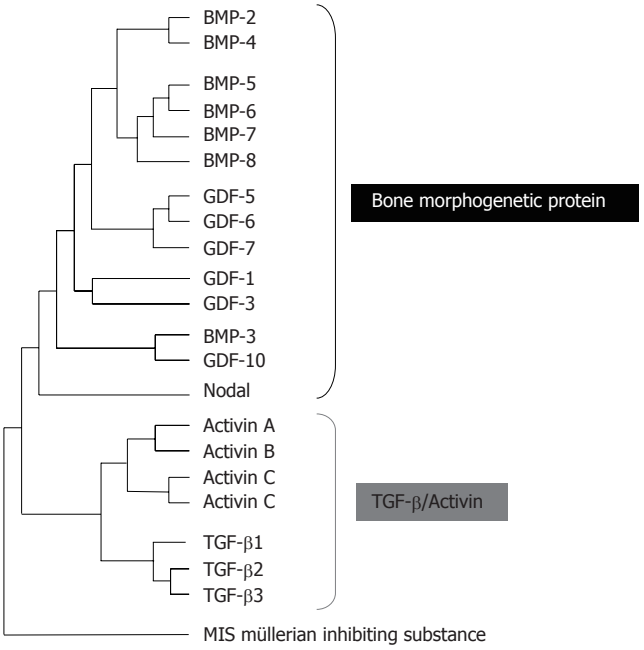

Figure 1 TGF-β family members.

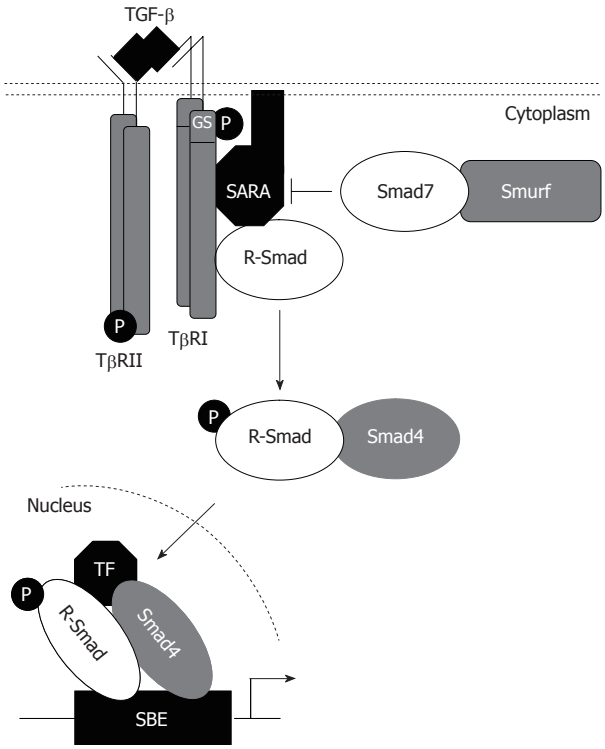

Figure 2 TGF-β/Smad signalling pathway.

physical interactions of the LAP with other proteins, such as thrombospondin-1[11].

TGF-β receptors

Signaling by TGF-β family members occurs through type Ⅰ (TβRI) and type Ⅱ (TβRⅡ) receptors (Figure 2). Five type Ⅱ and seven type I receptors, termed Activin-receptor-like kinases (ALKs) have been identified in vertebrates[12]. TβRI and TβRⅡ are similar transmembrane serine/threonine kinases, but type I receptors have a conserved Gly/Ser-rich (GS box) upstream from the kinase domain. In the absence of ligand, TβRI and TβRⅡ are present as homodimers in the plasma membrane[13]. Ligand binding induces the assembly of type I and type Ⅱ receptors into complexes, within which TβRⅡ phosphorylates and activates TβRI. This phosphorylation event is associated with activation of TβRI kinase and subsequent downstream signalling[12].

TGF-β SIGNALLING BY SMAD PROTEINS

Smad proteins

Signaling from activated TβRI to the nucleus occurs predominantly by phosphorylation of cytoplasmic protein mediators belonging to the Smad family[9]. The receptor-associated Smads (R-Smads; Smad1, 2, 3, 5 and 8) are recruited to activated TβRI by auxiliary proteins such as Smad Anchor for Receptor Activation (SARA)[14]. They all consist of two conserved Mad-homology (MH) domains that form globular structures separated by a linker region[15]. The N-terminal MH1 domain has DNA-binding activity, whereas the C-terminal MH2 domain has protein-binding and transactivation properties. Upon phosphorylation by activated TβRI on two serine residues within a conserved-SS(M/V)S-motif at the extreme C terminus, activated R-Smads form heteromeric complexes with a Co-Smad, Smad4, and are translocated into the nucleus where they may function as transcription factors directly or in association with other DNA-binding factors[9,12,16]. Finally, the inhibitory Smads, Smad6 and Smad7, act in an opposing manner to R-Smads to antagonize signaling. They compete with R-Smads for binding to activated TβRI and thus inhibit the phosphorylation of R-Smads and/or recruit E3-ubiquitin ligases to activated TβRI, resulting in receptor degradation[16]. Additionally, they may recruit protein phosphatase-1 (PP1) to the receptor complex, resulting in the dephosphorylation, thus inactivation, of the receptors via the catalytic subunit of PP1, GADD45[9]. Once in the nucleus, Smad proteins activate transcription through physical interactions and functional cooperation of DNA-binding Smads with sequence-specific transcription factors and with the coactivators CBP and p300. The R-Smads MH1 domain can bind directly to DNA except in the case of Smad2 where a 30 amino acid insertion in this domain prevents DNA binding. The minimal Smad3/4-binding element (SBE) contains only four base pairs, 5'-AGAC-3', but there are reports of binding to other G/C-rich sequences[9,16,17].

TGF-β REGULATION OF EXTRACELLULAR MATRIX GENE EXPRESSION

The net accumulation of collagen in tissue fibrosis is a result of an imbalance between enhanced production and deposition and impaired degradation of ECM components, mostly collagens (Figure 3). To date, about 25 types of collagens have been identified. All collagen molecules consist of three polypeptides, so-called α-chains.

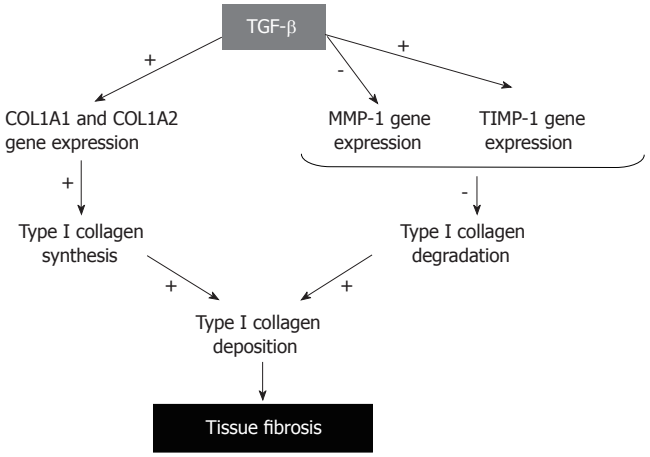

Figure 3 TGF-β, ECM gene expression and tissue fibrosis.

Some collagens are homopolymers with each of the three polypeptides being identical, while other collagens are heterotrimers with two or three distinct α-chains. Type I collagen, the major component of ECM is composed of two α1 (I) chains and one α2 (I) chain which are the products of two genes, *COL1A1* and *COL1A2*. After translation, the pro-α1 (I) and pro-α2 (I) polypeptides chains enter into the endoplasmic reticulum where specific proline and lysine residues are hydroxylated to form hydroxyproline and hydroxylysine. This event allows the pro-α chains to combine with other chains by hydrogen bonds and form the triple helix procollagen structure. Procollagens are then secreted through the Golgi apparatus in the extracellular space, where the N-terminal and C-terminal propeptides are cleaved by specific proteases. The mature processed collagen molecules aggregates to form larger collagens[18]. Abnormalities in any step of type I collagen production may result in abnormally elevated synthesis of type I collagen which, in turn, causes tissue fibrosis.

Several studies have shown that exaggerated tissue deposition of type I collagen during the fibrotic process is largely due to an increase in the rate of transcription of the corresponding genes[2,19,20]. To date, numerous efforts have been made to identify the signal transduction pathways involved in the transcription of type I collagen genes by TGF-β. Original works demonstrated that TGF-β-responsive sequences regarding the human promoter of *COL1A1* are located between 174 and 84 bp from the transcription start site, which region contains a binding site for Sp1 and an element with the canonical NF-1 binding motif[21]. Regarding the human *COL1A2* promoter, original works demonstrated that a 135-bp region of the promoter within 330-bp of the transcription start site could confer responsiveness to TGF-β[22,23]. The minimal TGF-β-response element was further refined to the region between nucleotides -271 and -235. The latter contains potential overlapping cis-element for Smad and AP-1, which are both implicated in *COL1A2* transactivation by TGF-β[24]. Several Sp1 binding elements contribute to basal gene expression, and may represent targets for anti-fibrotic intervention[25]. Cooperation between Smad3 and Sp1 to transactivate the *COL1A2* promoter have also been described, and it has been shown that Smad-p300/CBP interactions are critical for TGF-β driven *COL1A2* gene transactivation[26,27]. Other transcriptional coactivators such as SRC-1 may also participate in TGF-β effects[28].

By the end of the year 2000, only approximately 12 genes were known to contain Smad-responsive regions, binding Smad complexes directly or indirectly. All Smad gene targets identified downstream TGF-β were Smad3-dependent including *COL7A1*[29], *PAI-1*[30], and *COL1A2*[31]. Using a combined cDNA microarray promoter transactivation approach, we have identified new Smad3/4 gene targets in cultured dermal fibroblasts: *COL1A1, COL3A1, COL5A2, COL6A1, COL6A3*, and *TIMP-1*. In addition, we identified 49 immediate-early TGF-β target genes. Their activation by TGF-β is rapid and does not require protein neo-synthesis or JNK activity. Furthermore, their activation was blocked by overexpression of the inhibitory Smad, Smad7, and did not occur in Smad3-deficient mouse fibroblasts. Thus, we demonstrated that the Smad signaling pathway is crucial for simultaneous activation of skin fibrillar collagen genes (*COL1A1, COL1A2, COL3A1* and *COL5A2*) by TGF-β[32]. Besides playing a large part in the regulation of the expression of ECM components, Smads have been identified as capable of mediating the inhibitory activity of TGF-β on interstitial collagenase (matrix metalloproteinase-1, MMP-1) gene activation by pro-inflammatory cytokines, such as IL-1β[33], another mean by which the Smad pathway is likely to contribute to exacerbated ECM deposition.

TGF-β IN HUMAN SKIN FIBROSIS DISEASES

Keloids represent a dysregulated response to cutaneous wounding that results in an excessive deposition of collagen with a severely debilitating outcome for the affected patients. Several studies have demonstrated that TGF-β1 is expressed at greater levels in keloid fibroblasts when compared with normal dermal fibroblasts[1]. In addition, increased expression of TβRI and TBRⅡ, and increased phosphorylation of Smad3 in keloid fibroblasts, have also been reported[34], supporting the hypothesis that TGF-β/Smad signaling plays a central role in keloid pathogenesis. Furthermore, the activation of Smad signaling, importantly that of Smad3, appears to be one facet of the complex epithelial-mesenchymal interactions in keloid pathogenesis, resulting in active keratinocyte proliferation and collagen production by fibroblasts[35].

Skin tissue fibrosis may also be a sequel of both radiotherapy or accidental exposure to gamma irradiation[36]. Superficial fibrosis is a sequel in humans after radiotherapy[37], and is characterized by induration of the dermis and the subcutaneous tissue. In cases of radiation accidents, high doses of radiation can be delivered to the skin and severe skin burns can be observed, resulting in the development of extensive fibronecrotic tissues[36,38]. The concept concerning the initiation of radiation damage proposes that a cascade of cytokines is initiated

immediately after irradiation, during the clinically silent period, persists for long periods of times, and leads to the development of late damage[39]. The involvement of TGF-β in this early cascade has been reported in various irradiated tissues including skin, intestine, mammary gland and lung[36]. For example, in skin fibrotic samples from soldiers that suffered accidental irradiation in Lilo, Georgia, 1997, gene expression studies for collagen type Ⅰ and Ⅲ, and TGF-β1 showed that these three genes are specifically overexpressed. In addition, TGF-β1 protein was overexpressed in fibronecrotic skin both in the scar epidermis and in the fibrotic dermis[36].

Systemic sclerosis (SSc) is a heterogeneous and generalized connective tissue disorder characterized by micro-vascular and larger vessel lesions, with consequent induration and thickening of the skin, fibrotic degenerative changes in muscles, joints and viscera, mainly the intestinal tract, the heart, the lungs and the kidneys. Although the mechanisms involved in the pathological increase of collagen expression in SSc have not been entirely elucidated, extensive recent efforts have been devoted to study the role of TGF-β signaling pathway by Smad proteins[40]. Immunohistochemical analysis of skin biopsies performed in non lesional areas from SSc patients and analysis of fibroblast cultures showed that Smad2 and Smad3 expression and their nuclear translocation were increased in these SSc patients[41]. More recently Dong *et al*[42]. reported reduction of Smad7 expression in SSc derived fibroblast cultures as compared to fibroblast cultures from unaffected areas of the same patients, suggesting that a defective Smad7 feedback inhibition could play a role in TGF-β hyper-responsiveness in SSc.

TGF-β has also been implicated as being a key mediator in a number of fibrotic diseases in organs other than skin. For example, an increased expression for TGF-β has been documented during the phase of tissue remodeling in several forms of acute or chronic lung disease[43], such as rapid progressive pulmonary fibrosis[44], idiopathic pulmonary fibrosis[45], scleroderma[46], or cystic fibrosis[47]. In the cardiovascular system, mounting evidence supports the notion that TGF-β1 stimulates the progression of cardiac fibrosis during cardiac hypertrophy and heart failure[48]. In the kidney, TGF-β is closely associated with renal interstitial fibrosis, in which normal glomerular tissue is replaced by ECM, leading to organ failure[49]. Epithelial-to-Mesenchymal transdifferentiation induced by TGF-β may contribute to tubular atrophy and generation of interstitial myofibroblasts, leading to concomitant tubulo-interstitial fibrosis[50]. In advanced liver fibrosis resulting in cirrhosis, liver failure, and portal hypertension, TGF-β fibrotic action is broadly associated with its ability to lead transdifferentiation of hepatic stellate cells into myofibroblasts[51].

Smad3, A KEY MEDIATOR OF FIBROTIC PROCESSES

The most direct evidence supporting the involvement of Smad3 in fibrosis came from the use of mice with a targeted deletion of *Smad3*[52]. For example, skin from *Smad3*$^{-/-}$ mice exposed to a single dose of 30 to 50 Gy of gamma-irradiation showed significantly less epidermal acanthosis and dermal influx of mast cells, macrophages, neutrophils and decreased expression of TGF-β than skin from wild type littermates suggesting that inhibition of Smad3 could decrease tissue damage and reduce fibrosis after exposure to ionizing radiations[53]. In another experimental model of fibrosis, mice deficient in Smad3 exhibited suppressed type I procollagen mRNA expression and reduced hydroxyproline content in the lungs compared with wild-type mice treated with bleomycin. Furthermore, loss of Smad3 greatly attenuated morphological fibrotic responses to bleomycin in the mouse lungs[54]. Likewise, transient overexpression of active TGF-β1 in lungs, using adenoviral vector-mediated gene transfer, resulted in progressive pulmonary fibrosis in wild-type mice, whereas no fibrosis was seen in the lungs of *Smad3*$^{-/-}$ animals[55]. Conversely, C57BL/6 mice with bleomycin-induced lungs receiving an intratracheal injection of a recombinant adenovirus expressing Smad7 demonstrated suppression of type I procollagen mRNA, reduced hydroxyproline content, and no morphological fibrotic responses in the lungs, indicated that gene transfer of Smad7 prevents bleomycin-induced lung fibrosis[56]. More recently, using mice with targeted deletion of Smad3, Roberts *et al*[57]. demonstrated that lack of Smad3 prevents the epithelial-to-mesenchymal transition of lens epithelial cells following injury, and attenuates the development of fibrotic sequelae. Together, these various experimental approaches demonstrate the direct implication of Smad3 activation downstream of TGF-β in the pathogenesis of pulmonary fibrosis.

CONNECTIVE TISSUE GROWTH FACTOR

Although TGF-β has long been regarded as a pivotal growth factor in the formation and maintenance of connective tissues and as a major driving influence in many progressive fibrotic diseases, attention has recently focused on the role of connective tissue growth factor (CTGF) in fibrosis. For example, Systemic sclerosis (SSc) fibroblasts demonstrate constitutive over-expression of CTGF that promotes migration, proliferation and matrix production. Specifically, in fibroblasts cultured from SSc lesions, CTGF mRNA and protein are constitutively expressed, even in the absence of exogenously added TGF-β[58]. In normal adult fibroblasts, TGF-β induces the expression of CTGF *via* a functional Smad3 binding site in the CTGF promoter. However, mutation of the Smad binding site does not reduce the high level of CTGF promoter activity observed in dermal fibroblasts cultured from lesional areas of scleroderma patients. Thus, the maintenance of the fibrotic phenotype in scleroderma fibroblasts, as visualized by excess CTGF expression, appears to be independent of Smad-dependent TGF-β signaling[59]. The increased level of CTGF protein and mRNA is also associated with the accumulation of fibroblasts/myofibroblasts and collagen deposition in the persistence of late intestinal radiation fibrosis[60]. Interestingly, Balb/c mice that lack CTGF induction upon stimulation with bleomycin, can be transformed into fibrosis-sensitive individuals by generation of

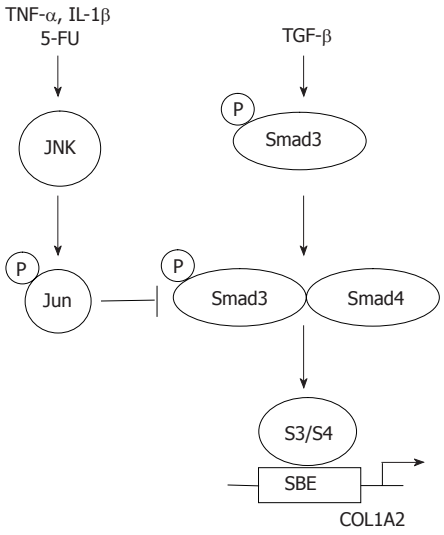

Figure 4 Inhibition of TGF-β-driven COL1A2 transcriptional activity by JNK.

a CTGF-rich environment using transient overexpression of CTGF by adenoviral gene transfer. In this context, silencing CTGF expression with siRNA demonstrated therapeutic potential to prevent liver fibrosis by inhibiting hepatic stellate cells activation[61]. Together these observations suggest that CTGF is an important mediator in the pathogenesis of fibrosis and can be act as an enhancer of TGF-β/Smad3 fibrotic response[62].

PERSPECTIVES FOR THERAPEUTIC INTERVENTION

Tremendous progress has been accomplished over the past several years in the understanding of the initial steps of TGF-β intracellular signalling. The identification of Smad proteins as direct links between the cell surface and the nucleus has allowed for the elucidation of critical events leading to gene activation by TGF-β. Specifically, an increasing body of evidence demonstrates that Smad3 plays a crucial role during the fibrotic process both *in vitro* and *in vivo*. These observations suggest that blocking the TGF-β/Smad3 pathways may promise opportunities for treatment of fibrotic diseases. In particular, several endogenous inhibitors of TGF-β/Smad3-mediated gene expression have been discovered. Firstly, Smad7 induction by IFN-γ, a well known anti-fibrotic cytokine, blocks TGF-β/Smad signalling pathway. In this context, halofuginone a low molecular weight plant alkaloid used as a coccidiostat for poultry, was effective in inhibiting dermal fibrosis in the tight skin mouse of scleroderma, and radiation-induced fibrosis[63-65]. Thus, halofuginone, which has demonstrated efficacy and tolerance in humans, could become an effective and novel therapy for example for liver fibrosis[66]. Secondly, activation of the MAP kinase JNK, whether by cytokines such as TNF-α or by pharmacologic molecules such as 5-fluoro-uracyl, blocks the transcriptional outcome of the TGF-β/Smad3 signaling pathway by induction of c-Jun phosphorylation which, directly interferes with Smad3-dependent transcription (Figure 4)[67-72]. Thirdly, cAMP was shown to inhibit TGF-β Smad3/4 dependent transcription via a protein kinase A-dependent mechanism[73]. However, several hurdles remain before the TGF-β/Smad3 pathway can be considered a perfect therapeutic target in situations such as fibrosis. The identification of alternate signalling pathways for TGF-β remains critically important. For example, the role of Smad2 downstream of TGF-β is rather poorly understood. Identification of Smad2 target genes will likely shed some light on alternate mechanisms by which TGF-β may affect connective tissue remodeling. Likewise, recent evidence for a role of the Rho pathway in the pathogenesis of radiation-induced enteritis suggest that inhibition of Rho pathway by pravastatin, an inhibitor of Rho isoprenylation, may also promise opportunities for new therapeutic perspectives[74].

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