

Localization of $\alpha\beta6$ integrin-TGF- β 1/Smad3, mTOR and PPAR γ in experimental colorectal fibrosis

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Abstract

A simultaneous action of several pro-fibrotic mediators appears relevant in the development of fibrosis. There are evidences that transforming growth factor- β (TGF- β)/Smad3 pathway forms with $\alpha\beta6$ integrin, mammalian target of Rapamycin (mTOR) and peroxisome proliferator-activated receptor- γ (PPAR γ) a complex signalling network with extensive crosstalk and strong effects on fibrosis development. The present study evaluated the expression of TGF β , Smad3, $\alpha\beta6$ integrin, mTOR and PPAR γ in 2, 4, 6-trinitrobenzenesulphonic acid (TNBS)-induced colorectal fibrosis in Smad3 wild-type (WT) and null mice. Smad3 WT mice treated with TNBS developed a marked colorectal fibrosis and showed a concomitant up-regulation of TGF β , Smad3, $\alpha\beta6$ and mTOR and a reduction of PPAR γ expression. On the other hand, Smad3 Null mice similarly treated with TNBS did not develop fibrosis and showed a very low or even absent expression of TGF β , Smad3, $\alpha\beta6$ and mTOR and a marked over-expression of PPAR γ . At the same time the expression of α -smooth muscle actin (a marker of activated myofibroblasts), collagen I-III and connective tissue growth factor (a downstream effector of TGF β /Smad3-induced extracellular matrix proteins) were up-regulated in Smad3 WT mice treated with TNBS compared to Null TNBS-treated mice. These preliminary results suggest a possible interaction between these pro-fibrotic molecules in the development of intestinal fibrosis.

Introduction

In inflammatory bowel disease (IBD), as

well as in other enteropathies, the chronic transmural damage elicits an excessive wound-healing response that may lead to fibrosis, strictures, stenosis and obstruction.¹⁻³ Intestinal fibrosis results from an abnormal response to a chronic local injury and is characterized by abnormal production and deposition of extracellular matrix (ECM) proteins produced by activated myofibroblasts, which are also called ECM-producing cells.⁴⁻⁷ These cells are derived not only from resident mesenchymal cells (fibroblasts, sub-epithelial myofibroblasts and smooth muscle cells), but also from epithelial and endothelial cells (by a process known as epithelial/endothelial-mesenchymal transition), stellate cells, pericytes, as well as intestinal or bone marrow stem cells.^{3,6,7} The most important soluble factors that regulate the activation of ECM-producing cells include cytokines, chemokines, growth factors, components of the renin-angiotensin system (RAS), angiogenic factors, peroxisome proliferator-activated receptors (PPARs), mammalian target of Rapamycin (mTOR), and products of oxidative stress.^{8,9} Other molecules, such as matrix metalloproteinases (MMPs) and specific tissue inhibitors of metalloproteinases (TIMPs), are also involved in regulating ECM turnover. Timing, concentration and sources of the main pro-fibrotic mediators might affect their individual contribution to tissue remodelling and fibrosis. Furthermore, a simultaneous action of some pro-fibrotic mediators appears relevant in the development of fibrosis.

Transforming growth factor- β (TGF- β) appears to play a central role in regulating the development, proliferation, differentiation and activation of intestinal mesenchymal cells, as well as in fibrosis.^{3-7,10}

All three mammalian isoforms of TGF- β (β 1, β 2, β 3) are expressed in the intestine that are synthesized and secreted in the latent form and must be activated before they can bind to their receptors and induce TGF β -mediated effects. TGF- β 1 is the most extensively studied and is considered the primary pro-fibrotic factor. Latency-associated protein (LAP) forms a non-covalent complex with TGF- β , termed latent complex which retains TGF- β in its inactive state until released.¹¹ Sequestration and regulated release of active TGF- β from LAP in this complex provide a mechanism by which the biologic function of TGF- β is controlled at the cellular level. Latent TGF- β 1 can be activated by both proteolytic and non-proteolytic mechanisms.^{11,12} Several proteases can release TGF- β 1 from LAP- β 1 including plasmin, urokinase-type and plasmin activators, tissue-type plasminogen activators, matrix metalloproteinases 2 and 9, and cathepsin.¹² Non-proteolytic activation involves the interaction of LAP- β 1 with another protein and induc-

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tion of a conformational change thereby activating TGF- β 1. LAP- β 1 can bind to any of the $\alpha\beta$ -containing integrins, but not all integrins that bind LAP- β 1 activate latent TGF- β 1.¹¹ Integrins are heterodimeric transmembrane proteins made up of α and β subunits. Six, of the 24 currently described integrins are able to bind the RGD motif in the LAP of TGF- β . Four of these integrins ($\alpha\beta3$, $\alpha\beta5$, $\alpha\beta6$ and $\alpha\beta8$) are thought to be able to activate TGF- β . The role of integrin-mediated TGF- β activation *in vivo* has only been confirmed for the $\alpha\beta6$ and $\alpha\beta8$ integrins.¹¹

Once activated, TGF- β 1 binds to specific membrane receptors (TGF- β RI, TGF- β RII, TGF- β RIII) leading to activation of intracellular transduction pathways. The canonical pathway is represented by Smad proteins.^{13,14} The activation of TGF- β receptors phosphorylates Smad2 and Smad3 which bind with the common mediated Smad4. The Smad2/3-Smad4 complex translocates into the nucleus where it regulates specific TGF- β target genes. TGF-beta signalling is negatively regulated by inhibitory Smad7. Besides Smads downstream pathways, TGF- β can also modulate, in a Smad/independent manner, other signal transduction pathways such ERK/cJUN/p38 MAP kinases and the phosphoinositide-3 kinase (PI3-K) and its downstream target Akt, also known as protein kinase B (PKB).¹³ Of the several fibrogenic molecules, $\alpha\beta6$ integrin, mTOR and PPAR- γ appear to interact directly with TGF- β /Smad pathway.

Integrins regulate cell-cell and cell-extracellular matrix interactions, thus influencing growth, differentiation, and development, as well wound healing and development of fibrosis.^{15,16} $\alpha\beta6$ is not expressed in normal condition, but it is up-regulated after tissue injury, in wound healing, in some types of epithelial

cancers and in many human fibrotic diseases of various organs (skin, lung, kidney and liver).¹⁶⁻²³ $\alpha\text{v}\beta\text{6}$ colocalizes with TGF β . $\alpha\text{v}\beta\text{6}$ ligands include fibronectin, tenascin, vitronectin and LAP. Interaction with LAP activates latent TGF- β and promotes fibrosis. $\alpha\text{v}\beta\text{6}$ integrin inhibitors significantly reduce tissue levels of profibrogenic transcripts, such as procollagen $\alpha\text{1(I)}$, αSMA , TGF β1 , TGF β2 , connective tissue growth factor (CTGF), TIMP-1 and $\alpha\text{v}\beta\text{6}$ integrin itself. Inhibition of the $\alpha\text{v}\beta\text{6}$ integrin, a key activator of TGF- β , could be an attractive therapeutic strategy for fibrosis, as it may be possible to inhibit TGF- β at sites of $\alpha\text{v}\beta\text{6}$ integrin up-regulation without affecting other vital homeostatic roles of TGF- β .

mTOR, a phosphatidylinositol 3-kinase-related kinase (PIKK), forms at least two distinct complexes.²⁴ The mTOR complex 1 (mTORC1) which is composed of mTOR, G protein beta subunit-like (G βL) and regulatory associated protein of TOR (Raptor) and control protein synthesis and cell growth and proliferation, as well as autophagy, angiogenesis and fibrosis. The mTOR complex 2 (mTORC2) consists of mTOR, G βL and Rapamycin-insensitive companion of TOR (Rictor) and is involved in the cell proliferation and survival, metabolic regulation and actin cytoskeleton organization. mTOR signalling is activated by hormones, growth factors, amino acid levels, stress and alterations in cellular energy status.²⁴ mTOR inhibitors (mTORis) exerts direct antifibrotic activities both by reducing the number of fibroblast and myofibroblasts and by down-regulating the production of fibrogenic cytokines, such as IL-4, IL-6, IL-13, IL-17, and TGF β1 , and the synthesis of type I and III collagen.²⁵⁻²⁷ Their antifibrotic effectiveness has been reported in fibrotic diseases of various organs including skin, lung, kidney, liver and intestine.²⁸⁻³³

PPARs are nuclear receptors, which regulate gene transcription by binding to retinoid X receptors (RXR) as functional heterodimers in response to a variety of endogenous and exogenous ligands.^{9,34} Three different isoforms of PPARs have been identified, termed PPAR α , PPAR γ and PPAR δ , each one encoded by specific genes. In particular the PPAR- γ isoform, identified mainly in the colorectal mucosa, but also in adipocytes, liver, vascular tissue and several inflammatory cells (monocytes and macrophages, dendritic cells, B and T cells) seems to be involved in several physiological processes, such as differentiation of adipocytes, glucose homeostasis, lipid metabolism, inflammatory and immune processes, as well as fibrosis.^{9,34} PPAR- γ activation seems to be strongly related to the TGF β /Smads pathway. The stimulation of PPAR- γ with specific ligands interferes with the Smad3 pathway by directly antagonizing Smad3 or down-regulat-

ing CTGF expression (a downstream effector of TGF β /Smad3-induced extracellular matrix proteins).^{9,35,36} There are evidences, therefore, that $\alpha\text{v}\beta\text{6}$, mTOR and PPAR γ form with TGF β /Smad3 pathway a complex signalling network with extensive crosstalk and strong effects on fibrosis development.

The aim of the present study was to evaluate the expression of TGF β , Smad3, $\alpha\text{v}\beta\text{6}$ integrin, mTOR and PPAR γ in 2, 4, 6-trinitrobenzenesulphonic acid (TNBS) induced colorectal fibrosis in Smad3 wild-type (WT) and null mice.

Materials and Methods

Animals

Twenty healthy adult mice, (Black Swiss \times 129SVJ strain) 5 weeks of age, were included in the study: 10 Smad3 wild-type (5 controls, 5 receiving TNBS) and 10 Smad3 null mice (5 controls, 5 receiving TNBS). All mice were maintained in a specific pathogen-free facility and routinely monitored. Mice were kept in microisolator cages and allowed free access to food and water.

The study protocol was approved by the Animal Research Committee of the University of LAquila, Italy.

Induction of colitis

Chronic colonic inflammation and fibrosis was induced in 5 Smad3 wild-type and 5 null mice, by weekly intra-rectal administration of TNBS (Sigma Aldrich, Milan, Italy) under light anaesthesia according to the method previously reported.³⁷ Each mouse received an incremental dose of TNBS over a 6-week period. At weeks I and II, mice received 0.5 mg of TNBS in 30% ethanol; at weeks III and IV, mice received 0.75 mg of TNBS in 45% ethanol; at weeks V and VI, mice received 1.0 mg of TNBS in 45% ethanol. The solution of TNBS-ethanol was administered in a total volume of 100 μL through a medical-grade polyurethane tube (diameter, 1 mm) the tip of which was positioned at 3 cm beyond the anus. Animals in the control groups (5 Smad3 wild-type and 5 null mice) received 100 μL of 0.9% saline instead of TNBS by enema. Animals were monitored daily for food and fluid intake and examined for signs of colitis including weight loss, diarrhoea, rectal bleeding and prolapse as well as signs of systemic inflammation such as piloerection, lethargy, and periorbital exudates.³⁷

Sample recovery and preparation

Laparotomy was performed under anaesthesia; the entire large bowel was rapidly excised and placed in a Petri dish containing sterile saline solution. The presence of adhesions

between the colon and adjacent organs was scored on a 0-2 scale.³⁷ The colon was then opened longitudinally, rinsed with sterile saline solution, weighed, measured, and then attached to a wooden tongue depressor for the assessment of macroscopic lesions. The colonic tissue samples were then fixed in 4% buffered formaldehyde and embedded in paraffin for histological and immunohistochemistry studies.

Assessment of macroscopic and microscopic colonic lesions

The sum of the scores of colonic lesions including adhesions, strictures, dilation, thickness, oedema/hyperaemia and ulcers was expressed as total macroscopic score (maximum score possible = 12).³⁷ Specimens obtained from the large bowel of all animals were washed and immediately immersed in 10% buffered formalin in phosphate buffer saline (PBS; pH 7.4) for 3 h at room temperature followed by the standard procedure for paraffin embedding. Serial 3- μm sections were stained with haematoxylin and eosin (H&E) to assess the degree of inflammation and with Masson's Trichrome to detect connective tissue and fibrosis. Stained sections were then observed under an Olympus BX51 Light Microscope (Olympus, Optical Co. Ltd, Tokyo, Japan). Intestinal fibrosis was scored as absent, mild, or severe, depending on the density and extent of Trichrome-positive connective tissue staining and disruption of tissue architecture.³⁷

Immunohistochemistry analysis

Colorectal specimens were promptly fixed with 10% buffered formalin in PBS (pH 7.4) for 3 h, dehydrated in graded ethanol, and embedded in a low-temperature fusion paraffin. Serial 3- μm sections were incubated for 40 min in methanol and 3% hydrogen peroxide solution and then rinsed in PBS. Thereafter, sections were incubated overnight at 4 $^{\circ}\text{C}$ with polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to $\alpha\text{-SMA}$ (sc-32251), Collagen I-III (sc-8784; sc-8781), CTGF (sc-14939), TGF β (sc-146), Smad3 (sc-6202), and PPAR γ (sc-7273), mTOR (Epitomics Inc., Burlingame, CA, USA; catalog 1612-1) used at a dilution of 1:100, 1:400, 1:200, 1:250, 1:100, 1:100 and 1:100, respectively, in PBS. Anti- $\alpha\text{v}\beta\text{6}$ integrin antibody (kindly provided by Biogen Idec, Cambridge, MA, USA) was used at a dilution of 1:100 in PBS.

Samples were then rinsed with PBS for 5 min and incubated with a labelled streptavidin-biotin-peroxidase conjugate kit (Dako LSAB, cod. K0675, Dako-Cytomation, Milan, Italy). After rinsing in PBS, for 10 min, the sections were incubated with 3,3-diaminobenzidine-

tetrahydrochloride (Sigma Aldrich) for 1-3 min. To control for specificity of the immune reaction, sections were incubated omitting the primary antibody (i.e. incubated only with the secondary antibody alone). Finally, samples were counterstained with Mayer's Haematoxylin and observed under a photomicroscope (Olympus BX51 Light Microscopy; Olympus, Optical Co. Ltd.).

Statistical analysis

Statistical analyses were performed using the Kruskal-Wallis non-parametric ANOVA system. *Post-hoc* comparisons between pairs of groups were assessed by using Wilcoxon rank sum test. Results were expressed as means \pm SD; a P-value <0.05 was considered statistically significant.

Results

Macroscopic findings

After TNBS treatment, the colons of Smad3 wild-type mice appeared, at macroscopic examination, significantly harder, thicker and shorter than those of the Smad3 null mice. The total macroscopic score was significantly higher in Smad3 WT mice treated with TNBS compared to that of Null TNBS-treated mice (5.40 0.89 *vs* 0.80 0.84, respectively, $P<0.05$).

Microscopic findings

In the untreated control mice, histological assessment showed normal morphological pattern and a similar connective tissue distribution both in Smad3 WT and null mice. In the TNBS-treated mice, marked changes were observed in the structure of the colon from Smad3 WT mice. The total microscopic score was significantly higher in Smad3 WT mice treated with TNBS compared to that of Null TNBS-treated mice (5.60 1.82 *vs* 1.80 0.84, respectively, $P<0.05$). A marked increase in connective tissue in the submucosa and serosa was found in Smad3 WT mice treated with TNBS compared to that in Null TNBS-treated mice (Figure 1). The degree of colonic fibrosis was significantly higher in WT treated with TNBS compared to that of Null TNBS-treated mice (1.60 0.55 *vs* 0.20 0.44, respectively, $P<0.05$).

Immunohistochemical evaluation

In WT control mice and in Null untreated and treated mice, α -SMA immunostaining, a marker of activated myofibroblasts, was localized in typical layers, while in the WT TNBS-treated mice α -SMA was more evident in muscularis mucosae, muscularis externa and it

was also present in submucosa and serosa layers (Figure 2).

In the untreated control mice, both WT and Null, collagen I-III and CTGF staining did not differ between the two groups and these were localized in the typical sides. In the WT TNBS-treated mice, collagen I-III and CTGF stainings were markedly increased in the *lamina propria*, submucosa and serosa layers compared to those of Null TNBS-treated mice (Figure 2).

TGF β 1 and Smad3 stainings, absent in WT and Null control mice and in Null TNBS-treated mice, were present in the submucosa and serosa of WT TNBS-treated mice (Figure 3). In the untreated control mice, both WT and Null, α v β 6 staining was absent. In Smad3 WT mice treated with TNBS, α v β 6 immunostaining was increased in submucosa and in serosa whereas it was absent in Smad3 Null TNBS-treated mice (Figure 3). In WT and control mice,

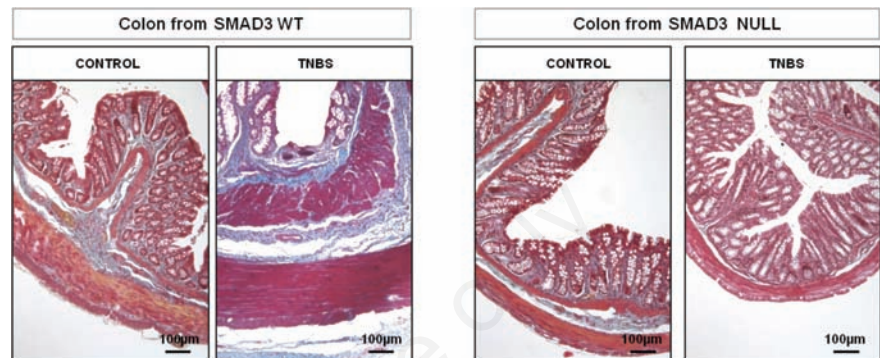


Figure 1. Masson's Trichromatic staining. Connective tissue distribution is similar in the two groups of control mice; in WT TNBS-treated mice a marked changes in colonic wall architecture due to abnormal deposition of connective tissue in *lamina propria*, submucosa and serosa were present, whereas the colonic wall of Null TNBS-treated mice is similar to that of untreated mice. Magnification: 10x.

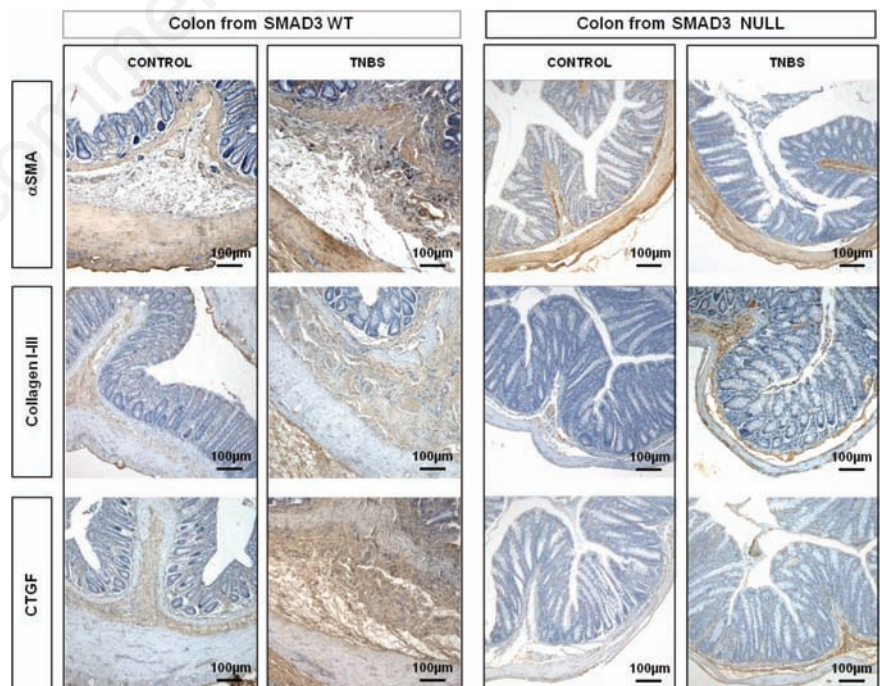


Figure 2. The α SMA expression is located in the typical areas (*muscularis mucosae* and *muscularis propria*) of Smad3 WT and Null control mice and in Smad3 Null TNBS-treated mice. Its expression is markedly increased in the colonic submucosa and serosa of Smad3 WT TNBS-treated mice. In TNBS-treated mice, collagen I-III and CTGF staining is markedly increased in *lamina propria*, submucosa and serosa layers from Smad3 WT mice compared to Null mice. Magnification: 10x.

mTOR was expressed in epithelial cells and in the apical portion of *lamina propria*, while it was absent in Smad3 Null control mice. mTOR staining was slightly increased in the epithelium and in submucosa and serosa layers of WT mice treated with TNBS compared to that of Null TNBS-treated mice (Figure 4). In Null mice treated with TNBS, PPAR γ staining was increased, both in the mucosa and in the submucosa layers, compared to WT TNBS-treated mice; its positivity was also more evident in Null control mice compared to WT controls (Figure 4).

Discussion

Intestinal fibrosis is a chronic and progressive process mediated by complex cell/matrix/cytokine and growth factor interaction,^{1,6-9} and TGF β has long emerged as a prominent regulator of fibrogenesis determining onset and progression of fibrosis in many chronic diseases. TGF β intracellular Smads transduction pathways appear to be crucial for development of fibrosis. Several studies have demonstrated that disruption of the TGF β /Smad3 signalling pathway by the loss of Smad3 confers resistant to tissues fibrosis in several organs including skin, kidney, lung and liver.³⁸⁻⁴² In a previous study we demonstrated that Smad3 Null mice are resistant to the development of experimental intestinal fibrosis induced by TNBS.³⁷ Histological and morphometric evaluation revealed a significantly higher degree of colonic fibrosis and accumulation of collagen in the Smad3 wild-type compared to null mice. Immunohistochemical evaluation showed a marked increase in α SMA, collagen I-III, CTGF, TGF β and Smad3 staining in the colon of Smad3 wild-type compared to null mice. All these findings have been confirmed by the present study.

In colonic fibrosis Smad3 could induce an abnormal activation of a fibrogenic phenotype of mesenchymal cells which turns into an increase of local deposition and accumulation of ECM proteins.^{1,5-7,9,10,37,43} The Smad2/3-Smad4 complex, by translocating into the nucleus, regulates specific pro-fibrogenic genes. Specifically, the target genes known to contain Smad-responsive regions and that are directly or indirectly involved in fibrogenesis, include several fibrillar ECM proteins (collagen, fibronectin), matrix-degrading enzymes (MMPs) and some specific inhibitors (TIMPs), as well as genes regulating epithelial-mesenchymal cell transition, proliferation (cyclin-dependent kinase inhibitor p21) and apoptosis (caspases).^{14,44}

In this study we have evaluated whether TGF β /Smad3 pathway and α v β 6 integrin,

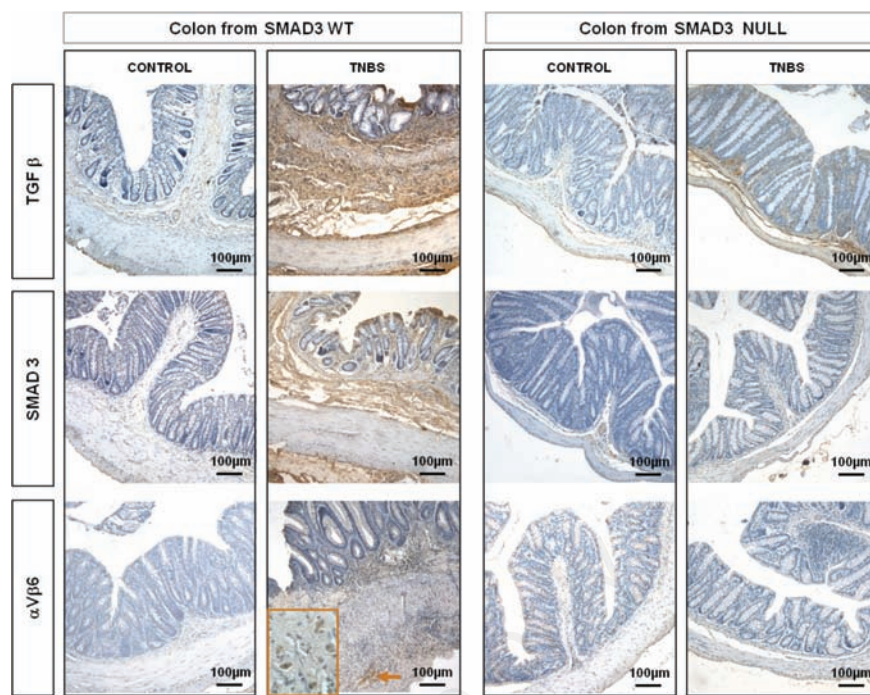


Figure 3. TGF β 1 and Smad3 stainings, absent in WT and Null control mice are markedly increased in *lamina propria*, submucosa and serosa layers in colon of Smad3 WT mice treated with TNBS compared to Null TNBS-treated mice. α V β 6 immunostaining is increased in submucosa in Smad3 WT TNBS-treated mice, whereas it is absent in Smad3 Null TNBS-treated mice. Magnification: 10x.

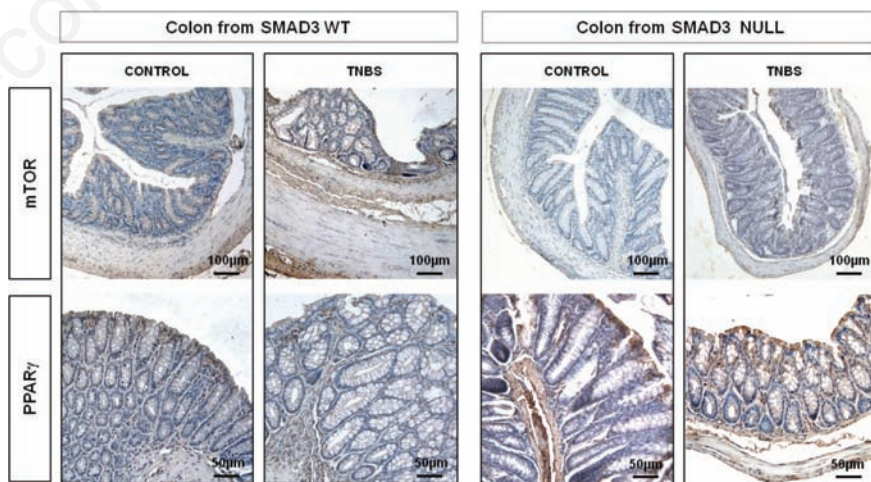


Figure 4. mTOR immunostainin: in the untreated WT and Null control mice mTOR is expressed in the epithelium and in the apical portion of *lamina propria*; mTOR staining is slightly increased in the epithelium, submucosa and serosa layers of Smad3 WT mice treated with TNBS compared to that of Smad3 Null TNBS-treated mice. Magnification: 10x. PPAR γ immunostaining: overall, PPAR γ staining is more marked in Smad3 Null mice, both in treated and untreated mice; it increased, both in the mucosa and submucosa layers, in Smad3 Null mice treated with TNBS compared to that of Smad3 WT TNBS-treated mice. Magnification: 20x.

mTOR and PPAR γ may interact in TNBS-induced colorectal fibrosis in Smad3 WT and Null mice. Smad3 WT mice treated with TNBS developed a marked colorectal fibrosis and showed a concomitant up-regulation of TGF β , Smad3, α v β 6 and mTOR and a reduction of PPAR γ expression. On the other hand, Smad3 Null mice similarly treated with TNBS did not develop fibrosis and showed a very low or even absent expression of TGF β , Smad3, α v β 6 and mTOR and a marked overexpression of PPAR γ . At the same time the expression of α SMA (a marker of activated myofibroblasts), collagen I-III and CTGF (a downstream effector of TGF β /Smad3-induced ECM) were up-regulated in Smad3 WT mice treated with TNBS compared to Null TNBS-treated mice. These preliminary data suggest a possible interaction between the above-mentioned molecules in the development of intestinal fibrosis, findings that need to be confirmed by *in vitro* studies using human intestinal fibroblasts or myofibroblasts cultures. To the best of our knowledge, this is the first report underlining the role of α v β 6 in the intestinal fibrosis, finding that may have important clinical implications in all human fibrogenic enteropathies, specially in the IBD.

The specific interaction between TGF β /Smad3 pathway and α v β 6 integrin, mTOR and PPAR γ is still unclear. TGF β isoforms are synthesized as latent molecules, consisting of mature TGF β that is covalently bound to the LAP.^{13,14} This latent complex associates with a family member of the latent TGF β binding proteins (LTBPs) that facilitates TGF β storage in the ECM. To be functional, TGF- β must be activated. There are several activators of TGF β that can dissociate the mature TGF β from LAP, allowing it to interact with its cell surface signalling receptors.^{11,12,15-17} Integrin-mediated activation seems to be the main mechanism of TGF β activation *in vivo*. Integrin α v β 6 can activate fibrogenic TGF β 1 through a mechanism that requires LTBP-1. α v β 6 is not expressed in normal condition, but it is up-regulated after tissue injury in epithelial cells of skin, kidney, lung, liver and intestine, as well as in many human fibrotic diseases of various organs including skin, kidney, lung and liver.^{11,12,15-23,45-47} α v β 6 can lead to local activation of TGF β 1 generating new active growth factor and then maintaining the TGF β mediated fibrotic process. Various genetic and pharmacologic interventions targeting the α v β 6 integrin have been shown to reduce the activation of TGF β 1 and fibrosis. Therefore, the α v β 6 blockade, could provide a new mechanism for injury specific attenuation of TGF β activity and fibrosis.

Given its pleiotropic effects, TGF β inhibition using strategies targeted to specific regions involved in fibrosis might be a better

alternative.¹⁸ Most other approaches are currently under consideration for targeting TGF β block either TGF β receptors or TGF β itself. These approaches might lead to unwanted side effects by interfering with important homeostatic effects of TGF β at sites outside the organs affected by tissue fibrosis. Mice deficient in TGF β 1 exhibit uncontrolled tissue inflammation, autoimmunity, and premature death, demonstrating a critical role for TGF β 1 in immune homeostasis. Although mice lacking α v β 6 do have mild inflammation in kidney, lung and skin, these effects are much less severe than those seen in mice lacking even a single TGF β isoform.¹⁸ Additionally, the α v β 6 integrin is highly upregulated in diseased tissue providing a mechanism for injury-induced TGF β activation as compared to homeostatic control of TGF β activity. By inhibiting a subset only of TGF β activation, particularly in injured epithelial organs, targeting α v β 6 could allow treatment of tissue fibrosis with substantially reduced risk of disrupting beneficial homeostatic control of inflammation and immunity.¹⁸

Extensive interaction also exists between mTOR and TGF β /Smads pathway which contributes to the proliferation of fibroblasts in many fibrotic disorders.²⁵ The activation of TGF- β receptors promote the phosphorylation of the PI3-K, which is a branch point for the activation of Akt. Once activated, Akt phosphorylates the tuberous sclerosis complex (TSC) that negatively regulates mTORC1.⁴⁸⁻⁵⁰ Thus active TSC is an inhibitor of mTORC1 and loss of TSC activity increase mTORC1 activity which induces fibroblast and myofibroblast proliferation.^{25,28} mTOR inhibitors reduce the myofibroblasts and down-regulate the production of TGF β 1, and the synthesis of type I and III collagen.²⁵⁻²⁷ Their antifibrotic properties have been reported in fibrotic diseases of several organs including skin, lung, kidney, liver and intestine.^{28-33,51,52}

PPAR- γ and TGF β /Smads pathway activities seems to be strongly related. PPAR- γ ligands may directly antagonize Smad3 or down-regulate CTGF expression that promotes the TGF-induced synthesis of collagen.^{9,35,36} PPAR γ agonists inhibit the fibroblast migration and proliferation⁵³ as well as the transdifferentiation of epithelial and mesenchymal cells in activated myofibroblasts,⁵⁴ one of the key points in fibrosis development. PPAR- γ ligands repress TGF β -induced myofibroblast differentiation and activation by targeting the PI3K/Akt and Smad3 pathways, respectively.^{55,56} Overexpression of PPAR γ prevents the development of tissue fibrosis, whereas its loss increases susceptibility to fibrosis.^{57,58} All these findings could explain the ability of PPAR γ to interfere in multiple phases of the tissue fibrotic processes. Therefore, PPAR γ should be regarded as an innate protection from exces-

sive fibrogenesis and a potential new target for the development of novel compounds with anti-fibrotic properties.⁵⁹ Several PPAR γ ligands with selective activity are under development. Experimental studies have shown that PPAR γ agonists attenuate fibrosis in various organs including lung, kidney, pancreas, liver and intestine, antifibrotic effects that are abolished by the use of a PPAR γ selective antagonists.⁶⁰⁻⁶⁵

Given all this, the data obtained suggest that the development of intestinal fibrosis could be influenced not only by TGF β -Smads signalling but also by α v β 6 integrin, mTOR and PPAR γ in a crosstalk integrated system. α v β 6 integrin may act by stimulating TGF β canonical (mediated by Smads) and non-canonical (mediated by mTOR) intracellular pathways. Increased expression of α v β 6 integrin, TGF β , Smad3 and mTOR is associated to the development of fibrosis, whereas up-regulation of PPAR γ appears to be protective towards fibrosis. Selective Smad3 disruption affects the expression of all these molecules and their effects on TNBS-induced colorectal fibrosis.

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