APPLICATION NOTE

HTRF tools for the successful investigation of tissue fibrosis.

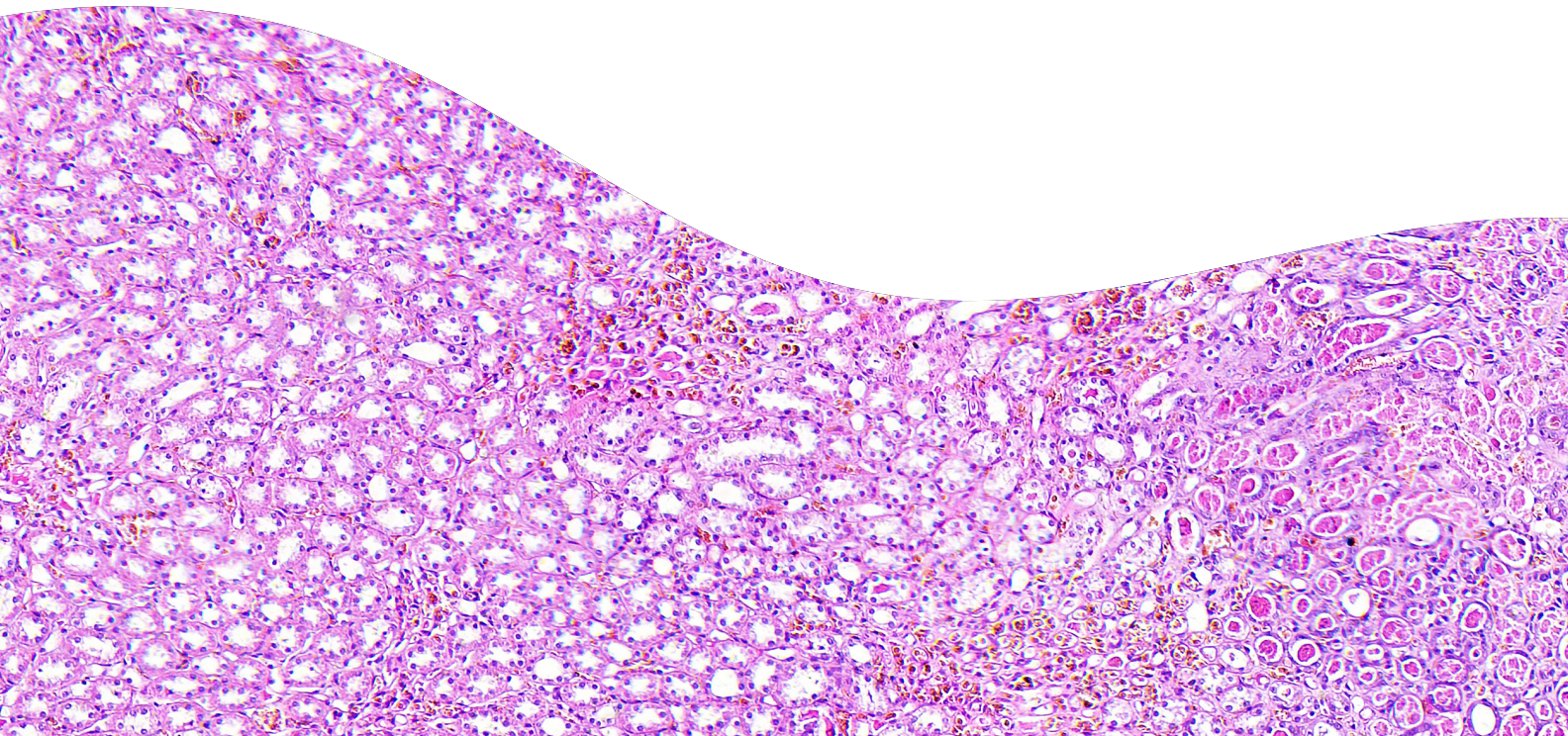
This note describes deep investigations of the molecular mechanisms involved in tissue fibrosis, and occurring in the major effector cell types.

Abstract

Fibrosis is an abnormal wound healing process in response to chronic tissue injury. It is defined as the replacement of healthy tissue by an excessive deposition of extracellular matrix (ECM), leading gradually to the disturbance and ultimately to the loss of organ architecture and function. It can occur in many tissues, such as in the four major organs: liver, lungs, kidneys and heart [1].

Tissue fibrosis represents a serious unresolved health problem resulting in increased morbidity and mortality. Because the detailed mechanisms behind this pathological state are still poorly understood, a deep investigation of the underlying cellular and molecular events is required to find therapies [1].

Numerous populations of cells and processes are involved in fibrogenesis. However, the activation of inflammatory cells and the accumulation of ECM-secreting myofibroblasts play a central role and represent common features of different types of fibrosis [2].



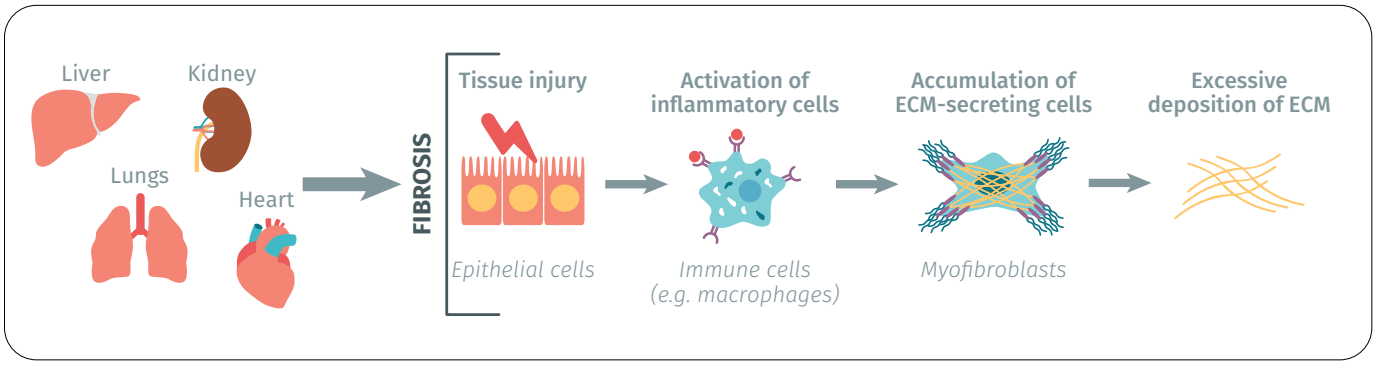


Figure 1: Cells and processes involved in fibrogenesis

To facilitate the study of tissue fibrosis and accelerate the identification of effective drugs, Revvity Bioassays has developed a platform of cell-based HTRF® (Homogeneous Time-Resolved Fluorescence) immunoassays dedicated to the analysis of key intracellular and secreted profibrotic proteins.

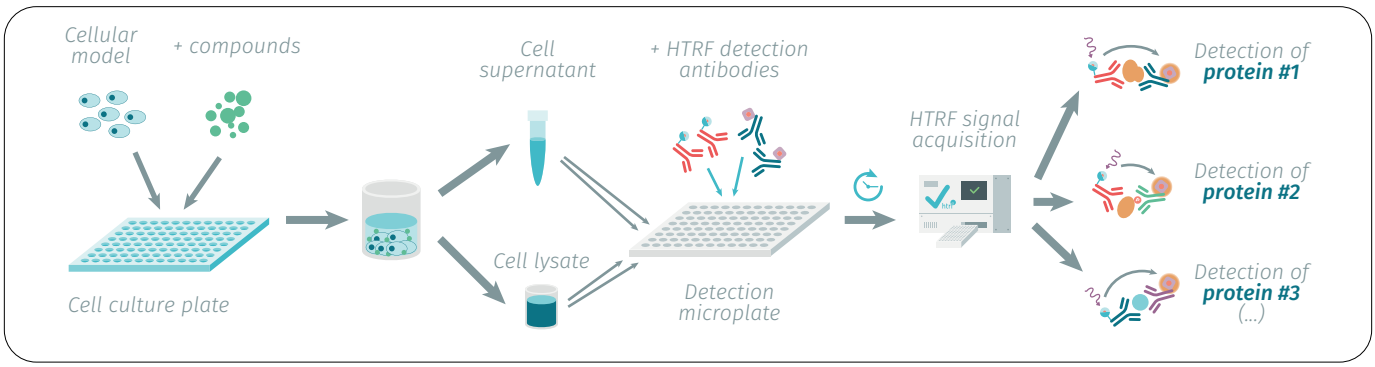


Figure 2: HTRF assay principle

Using illustrations from both hepatic and pulmonary fibrosis, this application note demonstrates the strength of the HTRF platform for the parallel measurement of numerous markers (phospho-/total proteins, cytokines, chemokines, ECM components...) on relevant cellular models.

1. Hepatic fibrosis

Overview

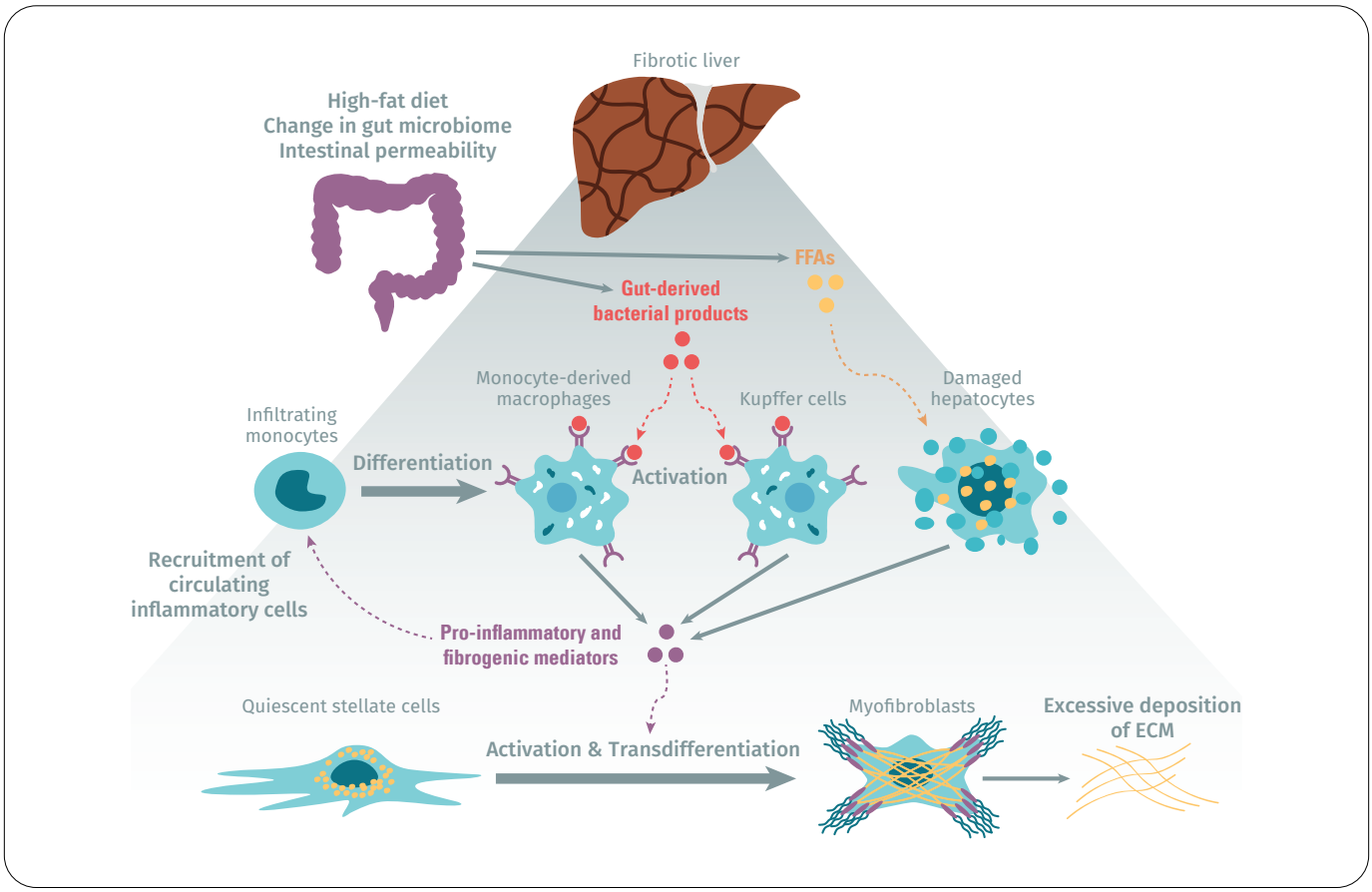


Figure 3: Overview of hepatic fibrosis

Hepatic fibrosis often occurs with Non-Alcoholic Steatohepatitis (NASH). Its progression results from numerous events originating within the liver, as well as from signals derived from the intestine:

- FFAs derived from high-fat diets accumulate within hepatocytes and are responsible for lipotoxicity. In response, damaged hepatocytes release pro-inflammatory mediators [1].
- Increased intestinal permeability and changes in gut microbiome contribute to the entry of gut-derived bacterial products into the liver, principally lipopolysaccharides (LPS). LPS activate the Toll-Like Receptor 4 (TLR4) on the surface of Kupffer cells (KCs) and stimulate the production of pro-inflammatory and fibrogenic molecules such as TGF-β1 [2].
- Pro-inflammatory chemokines (mainly CCL2) released by injured hepatocytes and activated KCs trigger the recruitment of circulating inflammatory monocytes to the liver, and their differentiation into profibrotic macrophages. These inflammatory cells are in turn activated by the overload of gut-derived bacterial products and also secrete pro-inflammatory and profibrotic factors [2].
- Pro-inflammatory and profibrotic mediators activate quiescent Hepatic Stellate Cells (HSCs) and trigger their transdifferentiation into proliferative and ECM-secreting myofibroblasts [3].

[1] Magee et al., Pathogenesis of Nonalcoholic Steatohepatitis: Interactions between Liver Parenchymal and Nonparenchymal Cells, BioMed Research International, Volume 2016, Article ID 5170402

[2] Tacke, Targeting hepatic macrophages to treat liver diseases, Journal of Hepatology 2017 vol. 66 j 1300–1312

[3] Tsuchida and Friedman, Mechanisms of hepatic stellate cell activation, Nature Reviews, Gastroenterology & Hepatology, Volume 14, July 2017

Investigation of the LPS/TLR4 axis in KCs

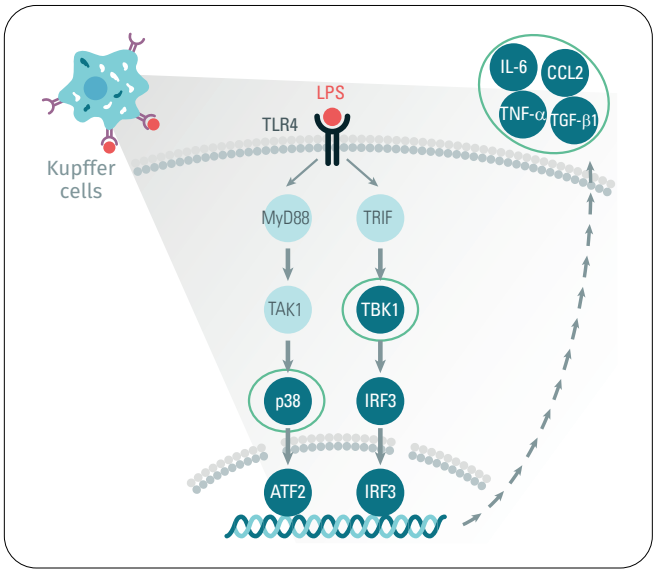


Figure 4: Kupffer cells in hepatic fibrosis molecular pathway

The mouse Kupffer cell line ImKC was plated in 96-well plates (100K cells/well) in complete medium with 10% serum. The day after, cells were incubated for 6h with only 4% serum before the addition of increasing doses of LPS diluted in serum-free medium. After a 1h or an overnight treatment, cell supernatants and lysates were collected.

The levels of intracellular phospho-p38 and phospho- & total TBK1 were analyzed using the dedicated HTRF assays 64P38PEG, 64TBKPEG, 64NTBPEG.
The concentrations of secreted TGF-β1, TNF-α, IL-6 and CCL-2 were measured using the corresponding HTRF kits 62HTGFBPEG, 62MTNFAPEG, 62MIL06PEG and 62MCCL2PEG.

- **Analysis of intracellular phospho-proteins**

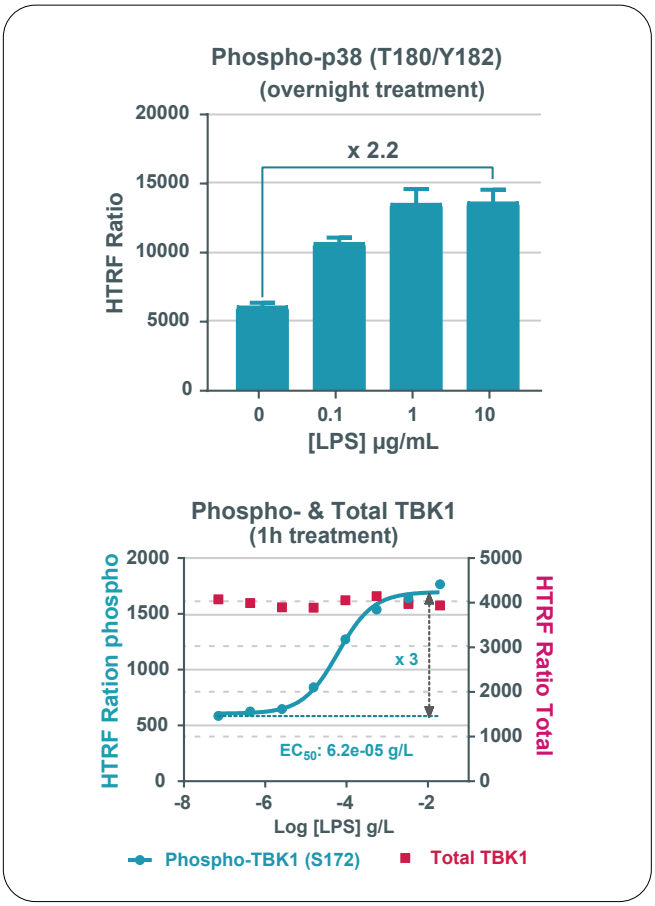


Figure 5: Phospho-p38 and Phospho/Total TBK1 analysis for Kupffer cells in hepatic fibrosis

As expected, LPS induces an increase in the phosphorylation levels of the kinases p38 and TBK1, highlighting the activation of TLR4 and its downstream MyD88- and TRIF-dependent signaling pathways.

- **Analysis of secreted cytokines**

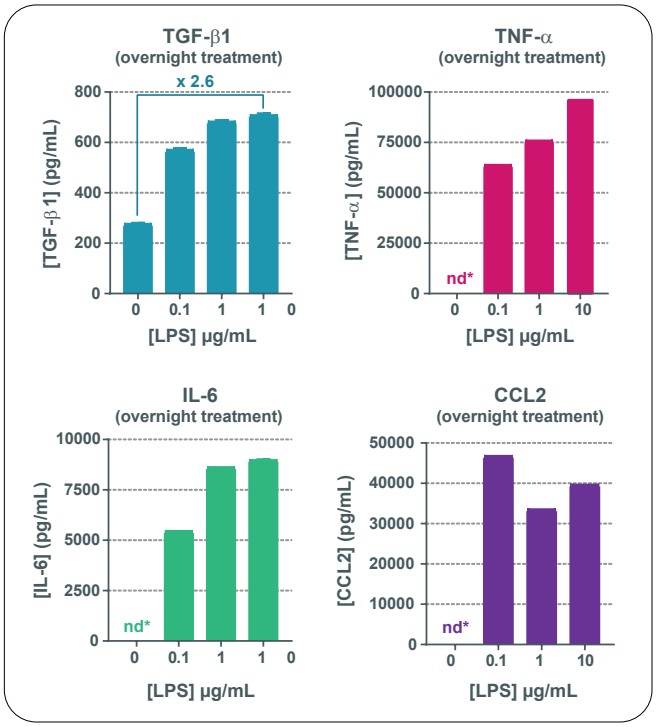


Figure 6: TGF-β1 , TNF-α , IL-6, and CCL2 analysis for Kupffer cells in hepatic fibrosis

*not detectable

Overnight exposure of Kupffer cells to LPS leads to a 2.6-fold increase in the secretion of the profibrotic mediator TGF-β1. LPS treatment also triggers the massive release of the pro-inflammatory cytokines TNF-α, IL-6, and CCL2, while non-treated cells produce non detectable concentrations.

These sets of data demonstrate the activation of Kupffer cells by LPS and their ability to secrete pro-inflammatory and fibrogenic factors.

Monitoring of signaling pathways involved in HSC activation

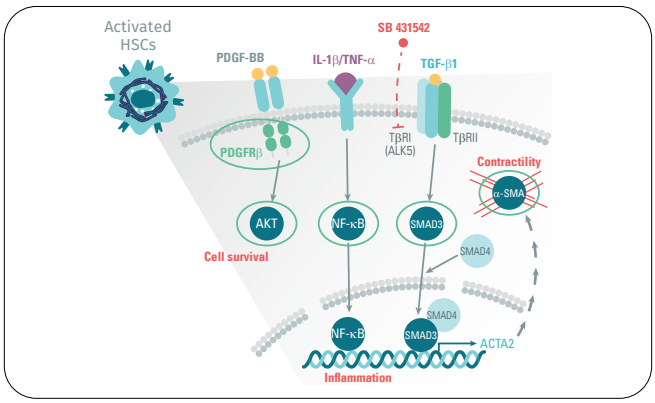


Figure 7: Activated HSCs in hepatic fibrosis molecular pathway

The human hepatic stellate cell line LX-2 (EMD Millipore, #SCC064) was plated in 96-well plates (50K or 100K cells/well) and incubated for 6h in complete medium before an overnight serum starvation step (with serum-free medium + 0.2% BSA). The day after, cells were treated for the desired time period with various compounds diluted in the starvation medium. Cell lysis was finally performed to measure the levels of phospho- & total SMAD3, α-SMA, phospho-PDGFRβ, phospho- & total AKT and phospho- & total NFκB using the dedicated HTRF assays 63ADK025PEG, 64ND3PEG, 62ASMAPEG, 64PDGPEG, 64AKSPEG, 64NKTPEG, 64NFBPEG, 64NFTPEG.

- **TGF-β/SMAD/α-SMA signaling**

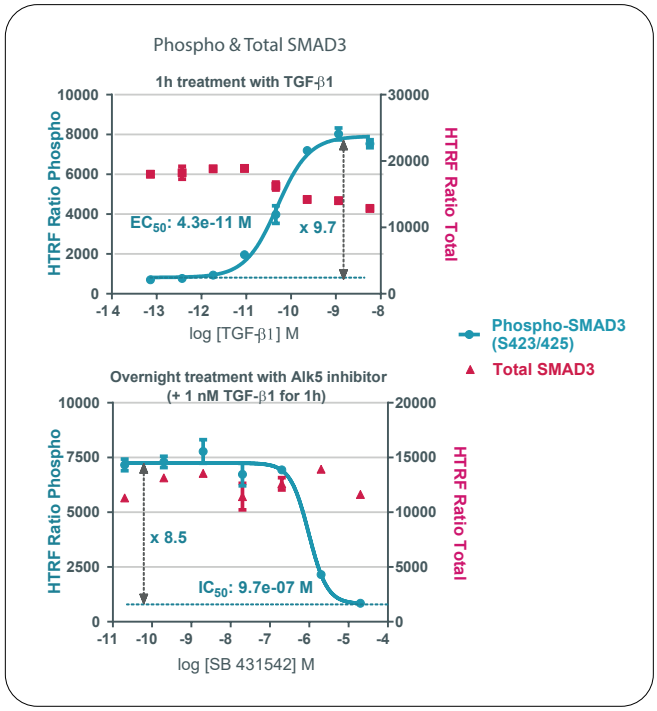


Figure 8: Phospho/Total SMAD3 analysis for Activated HSCs in hepatic fibrosis

After a 1h treatment, TGF-β1 induces a 10-fold increase in SMAD3 phosphorylation without significant modulation of the expression level of the protein, demonstrating the proper activation of the TGF-β/SMAD signaling pathway in HSCs. Overnight incubation with the Alk5 inhibitor SB 431542 efficiently inhibits SMAD3 phosphorylation induced by the cytokine.

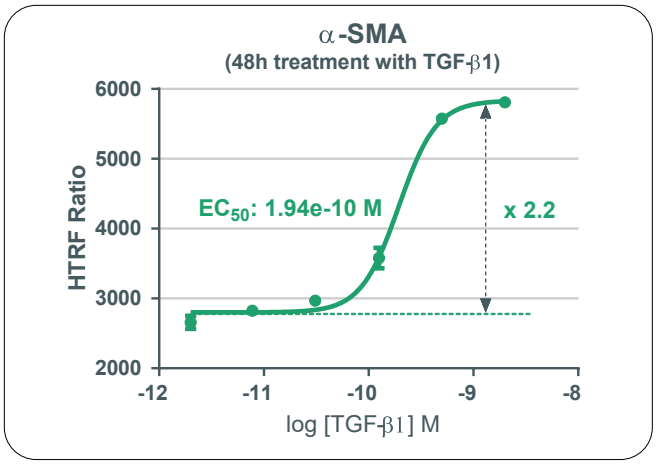


Figure 9: α-SMA analysis for Activated HSCs in hepatic fibrosis

TGF-β1 long-term treatment (48h) leads to a 2.2-fold increase in α-SMA expression level, highlighting the differentiation of HSCs into contractile myofibroblasts.

- **PDGF/AKT signaling**

PDGF-BB first triggers the phosphorylation of its receptor in a dose-dependent manner. Once activated, PDGFRβ initiates the PI3K/AKT signaling cascade, illustrated here by the strong increase in the phosphorylation of AKT on Ser473, while the total level of the protein remains stable.

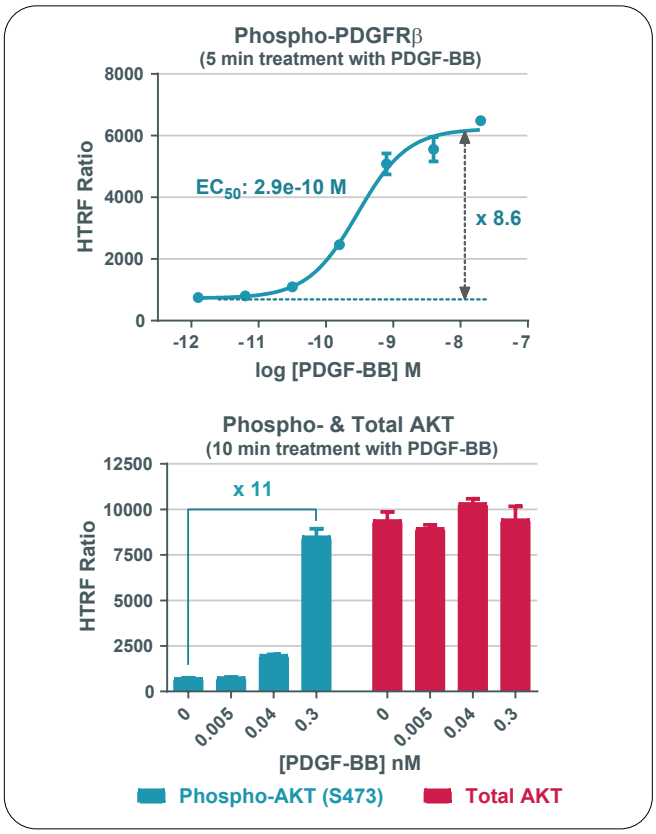


Figure 10: Phospho-PDGFRβ and phospho/Total AKT analysis for Activated HSCs in hepatic fibrosis

- **NFκB signaling**

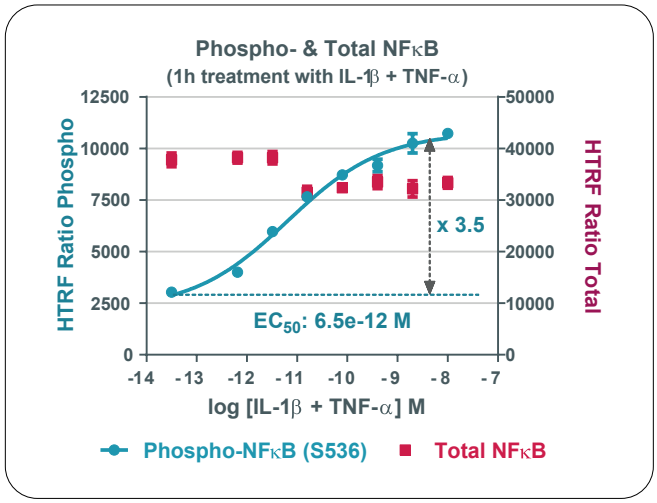


Figure 11: Phospho/Total NFκB analysis for Activated HSCs in hepatic fibrosis

LX-2 cell treatment with a cocktail of pro-inflammatory cytokines induces a 3.5-fold increase in NFκB phosphorylation with no change in the expression level of the protein. These data demonstrate that IL-1β and TNF-α are responsible for the activation of NFκB, which is a critical mediator of inflammatory responses in hepatic stellate cells.

Quantification of ECM molecules secreted by myofibroblasts

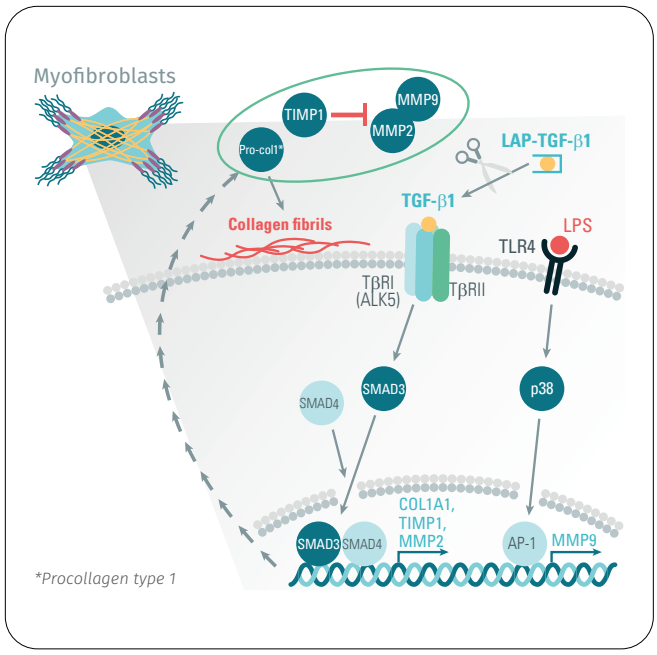


Figure 12: Myofibroblasts in hepatic fibrosis molecular pathway

The LX-2 cell line (EMD Millipore, #SCC064) was plated in 96-well plates (50K or 100K cells/well) in complete medium. The day after, cells were treated with increasing doses of TGF-β1 or LPS diluted in serum-free medium with 0.2% BSA. After different incubation time periods (overnight to 48h), cell supernatants were collected and their concentrations of pro-collagen type 1 (HTRF assay 63ADK014PEG), TIMP1*, MMP2* and MMP9* were quantified.

*New HTRF assays for release in October 2018

- **TGF-β1 treatment**

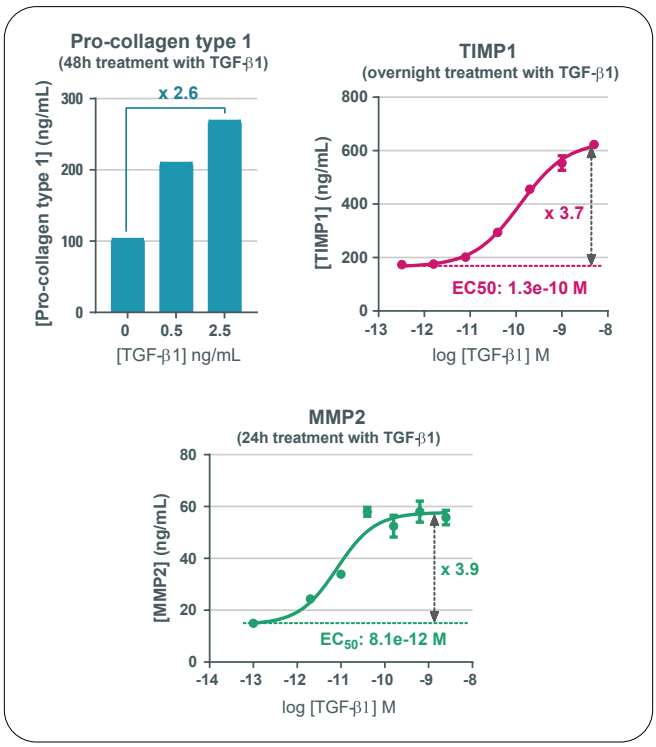


Figure 13: Pro-collagen type 1, TIMP1 and MMP2 analysis for myofibroblasts in hepatic fibrosis

The profibrotic cytokine TGF-β1 induces the activation of the transcription factor SMAD3, responsible for the expression and secretion of pro-collagen type 1. This ECM molecule is then processed into mature collagen which accumulates in the extracellular space and participates in ECM deposition.

In hepatic fibrosis, the ECM-regulators MMP2 and TIMP1 are also key fibrogenic markers upregulated by TGF-β1 signaling.

- **LPS treatment**

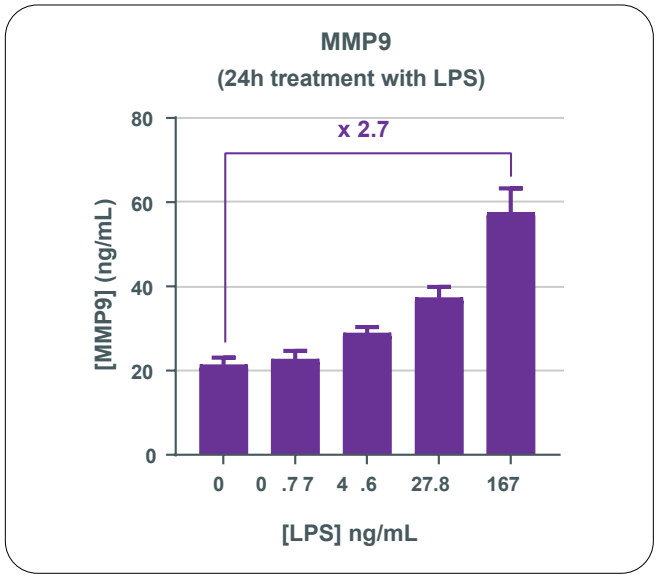


Figure 14: MMP9 analysis for myofibroblasts in hepatic fibrosis

Cell treatment with LPS for 24h induces a 2.7-fold increase in the secretion of MMP9. This matrix metalloproteinase is involved in the proteolytic activation of TGF-β1, and therefore represents another important fibrogenic marker.

2. Pulmonary Fibrosis

Overview

Pulmonary Fibrosis (PF) is a chronic respiratory disorder characterized by a scarring and thickening of lung tissue. This damage causes irreversible loss of the ability to transport and exchange oxygen, and eventually leads to death. When an etiology for PF cannot be clearly identified, the condition is termed Idiopathic Pulmonary Fibrosis (IPF) [1].

IPF results from recurrent injury to epithelial cells triggered by a variety of chemical and environmental exposures, but also by infections, auto-immune diseases and genetic predisposition. In response, damaged epithelial cells release pro-inflammatory mediators that recruit and activate circulating immune cells as well as lung-resident macrophages. Inflammatory cells in turn secrete profibrotic factors (mainly TGF-β1), which induce the transdifferentiation of lung fibroblasts into contractile and ECM-secreting myofibroblasts [2].

[1] Brown, A Review of Pulmonary Fibrosis, US Pharm. 2015;40(7):HS12-HS16

[2] Renzoni et al., Pathogenesis of idiopathic pulmonary fibrosis: review of recent findings, F1000Prime Reports 2014, 6:69 (doi:10.12703/P6-69)

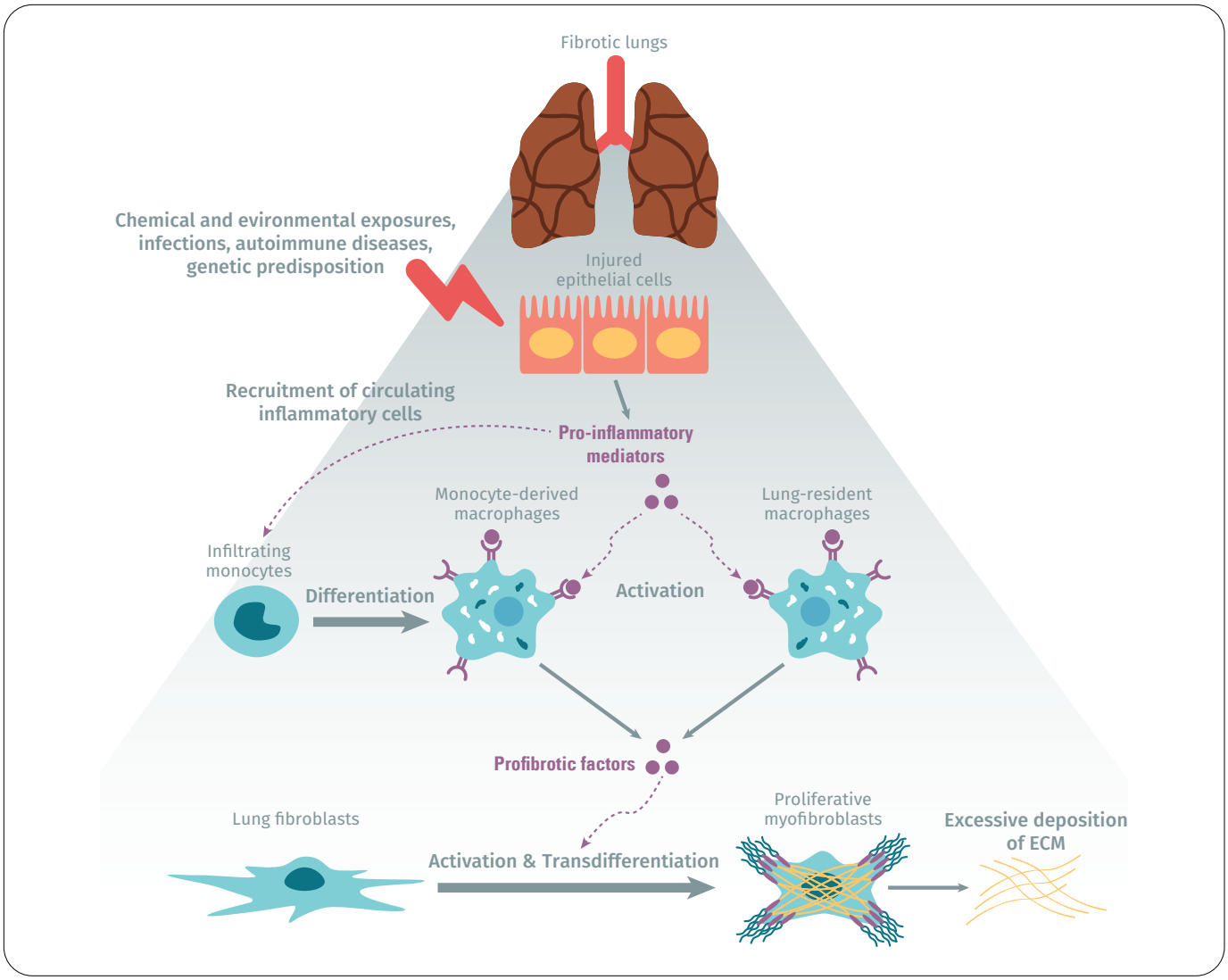


Figure 15: Overview of pulmonary fibrosis

Investigation of the TGF-β/SMAD/α-SMA axis in lung fibroblasts

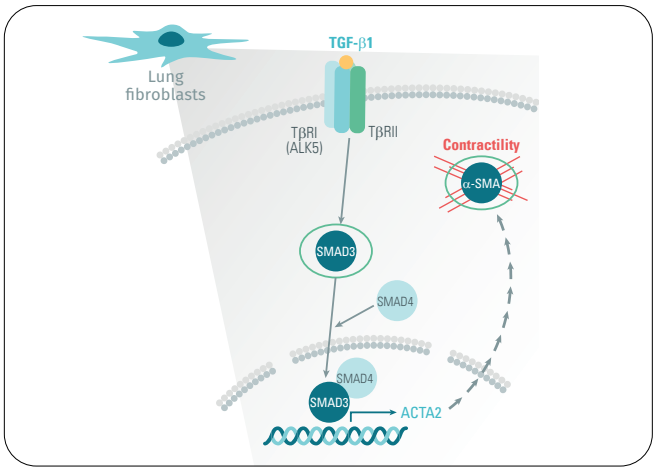


Figure 16: Lung fibroblasts in pulmonary fibrosis molecular pathway

The human lung fibroblast cell line MRC-5 was plated in a 96-well plate (30K cells/well) and incubated for 6h in complete medium before an overnight serum starvation step (with serum-free medium + 0.2% BSA).

The day after, cells were treated for 1h or overnight with increasing doses of TGF-β1, and then lysed to analyze the levels of phospho- & total SMAD3 and α-SMA using the dedicated HTRF assays 63ADK025PEG, 64ND3PEG and 62ASMAPEG.

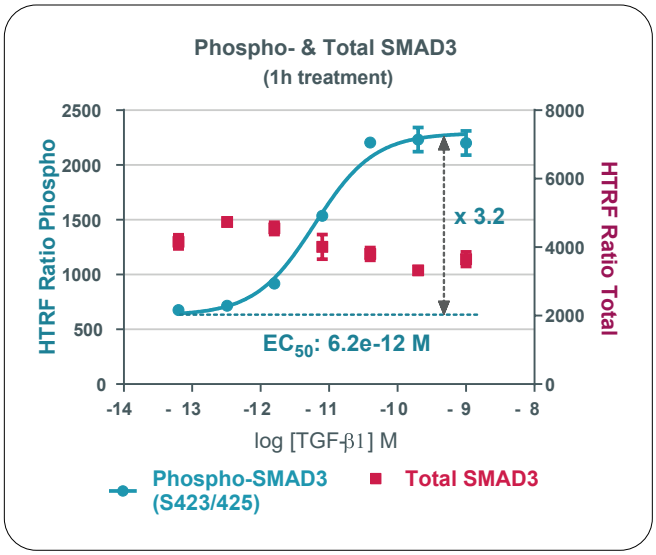


Figure 17: Phospho/Total SMAD3 analysis for lung fibroblasts in pulmonary fibrosis

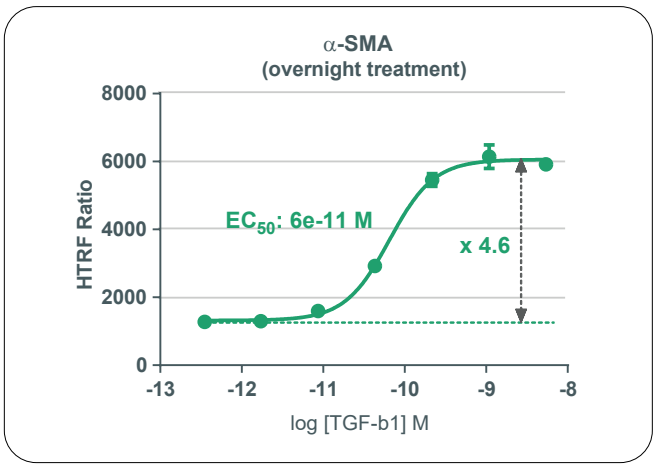


Figure 18: α-SMA analysis for lung libroblasts in pulmonary fibrosis

Cell treatment with TGF-β1 for 1h induces a 3.2-fold increase in SMAD3 phosphorylation without a significant change in the total level of the protein, highlighting the proper activation of the TGF-β/SMAD signaling pathway in lung fibroblasts.

In addition, the overnight exposure of cells to the profibrotic cytokine leads to a strong dose-dependent increase in the α-SMA expression level, which is a key marker of the transdifferentiation into contractile myofibroblasts.

Study of the release of ECM components and ECM-regulators by myofibroblasts

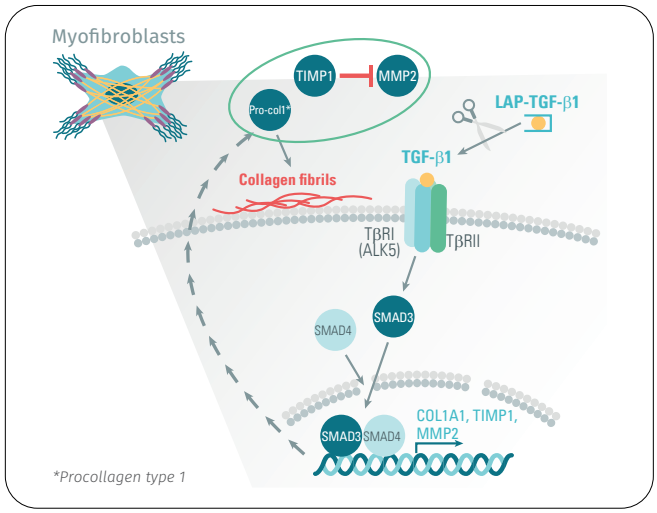


Figure 19: Myofibroblasts in pulmonary fibrosis molecular pathway

The human pulmonary fibroblast cell line MRC-5 was plated in a 96-well plate (30K cells/well) in complete culture medium. The day after, cells were treated with various concentrations of TGF-β1 diluted in serum-free medium with 0.2% BSA. After an overnight incubation, cell supernatants were collected and their pro-collagen type 1 (HTRF assay 63ADK014PEG), TIMP1, and MMP2 contents were measured.

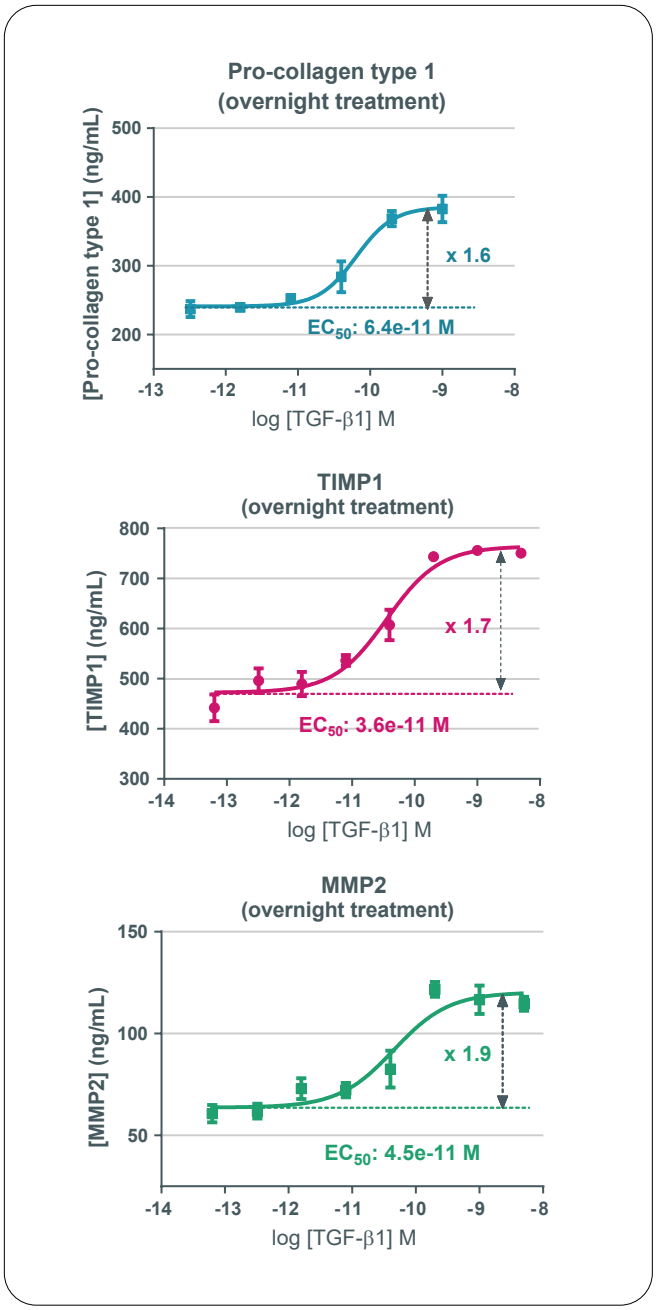


Figure 20: Pro-collagen type 1, TIMP1 and MMP2 analysis for myofibroblasts in pulmonary fibrosis

The fibrogenic mediator TGF-β1 triggers the activation of the SMAD3 signaling pathway, leading to the expression and secretion of pro-collagen type 1 by myofibroblasts derived from lung fibroblasts.

In pulmonary fibrosis, the matrix metalloproteinase MMP2 and the tissue inhibitor of metalloproteinase TIMP1 also constitute major fibrotic proteins that are upregulated by TGF-β1 signaling.

Conclusion

This application note describes deep investigations of the molecular mechanisms involved in tissue fibrosis, and occurring in the major effector cell types.

The useful sets of data obtained on hepatic and pulmonary cellular models highlight the added value of the HTRF platform for the easy, simultaneous and accurate analysis of numerous profibrotic markers on cellular samples.

Taken together, these assays enable a complete and precise insight into the cellular events leading to fibrosis, and they therefore facilitate the identification of new drugs to treat this major medical problem.

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