APPLICATION NOTE

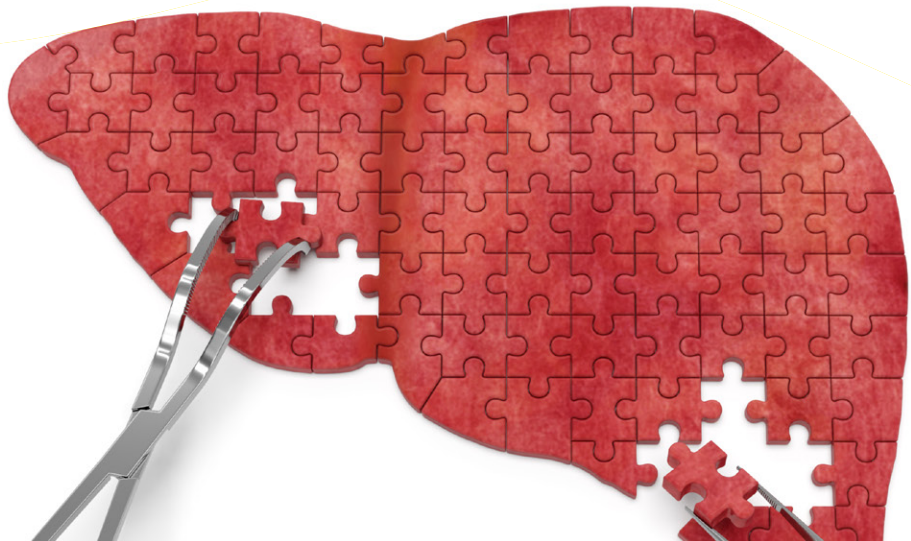
HTRF assays facilitate investigation of the TGF-β/SMAD/α-SMA signaling axis in liver fibrosis.

This application note explores the modulation of TGF-β1, SMAD3 and α-SMA in various NASH models using the HTRF® technology.

Abstract

Liver fibrosis occurs as a wound healing scar response following chronic liver inflammation, as found in NASH (Non-Alcoholic Steatohepatitis). Its progression is strongly associated with chronic activation of Kupffer cells (liver resident macrophages) and HSCs (Hepatic Stellate Cells), as well as with hepatocellular injury and subsequent inflammatory response.

Increased intestinal permeability and translocation of bacterial PAMPs (Pathogen-Associated Molecular Patterns) into the liver is a hallmark of chronic liver injury. Kupffer cells respond to LPS (lipopolysaccharide) through the Toll-like receptor TLR4 to produce various inflammatory mediators such as TGF-β, which is the main profibrotic cytokine. TGF-β/SMAD signaling in hepatocytes contributes to cell death, while it also drives the activation and trans-differentiation of HSCs into contractile and secretory myofibroblasts. Myofibroblasts produce large amounts of ECM proteins such as fibrillar collagen, leading to an excessive and irreversible ECM deposition. This pivotal cell type is characterized by de novo expression of α-SMA (α-Smooth Muscle Actin) which confers cell contractility.



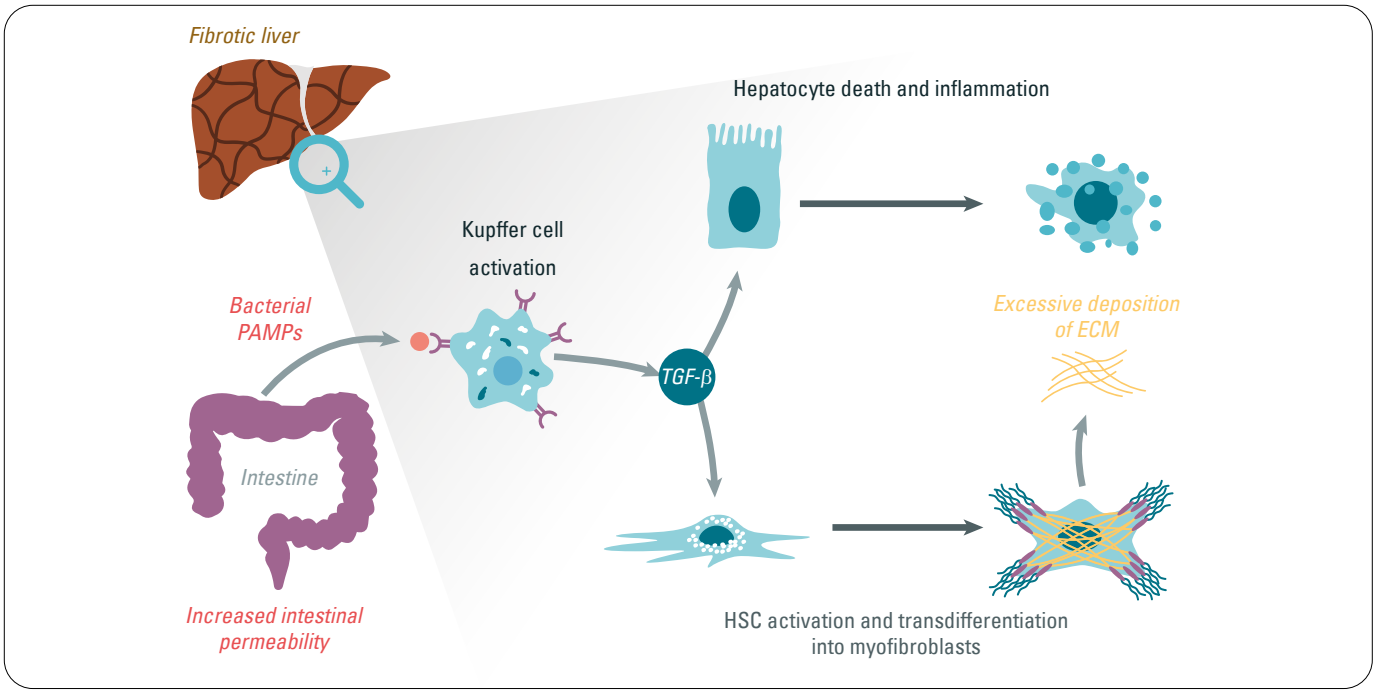


Figure 1: TGF- β implication in chronic liver inflammation progression

Because of its crucial role in the progression of this disorder, the TGF-β/SMAD/α-SMA axis has emerged as a therapeutic target. The availability of a platform of convenient, robust and sensitive assays to deeply investigate the main signaling events occurring in the different hepatic cell types is therefore of great interest. This application note illustrates the use of HTRF assays (homogeneous TR-FRET immunoassays) to analyze the status of the key molecular players involved in this signaling pathway.

TLR4 signaling and TGF-β release from Kupffer cells activated by LPS

The mouse Kupffer cell line ImKC was plated in a 24-well culture plate (650,000 cells/well) in complete culture medium with 10% serum and incubated for 5h before medium renewal with 4% serum. After an overnight incubation, the cells were treated for 16h with various concentrations of LPS diluted in medium with 4% serum.

Cell supernatants were collected and analyzed for their TGF-β1 concentration using the HTRF® TGF-β1 kit (62HTGFBPEG).

LPS treatment leads to an increase of TGF-β1, highlighting the activation of the TLR4 axis in these hepatic macrophages after exposure to gut-derived toxins.

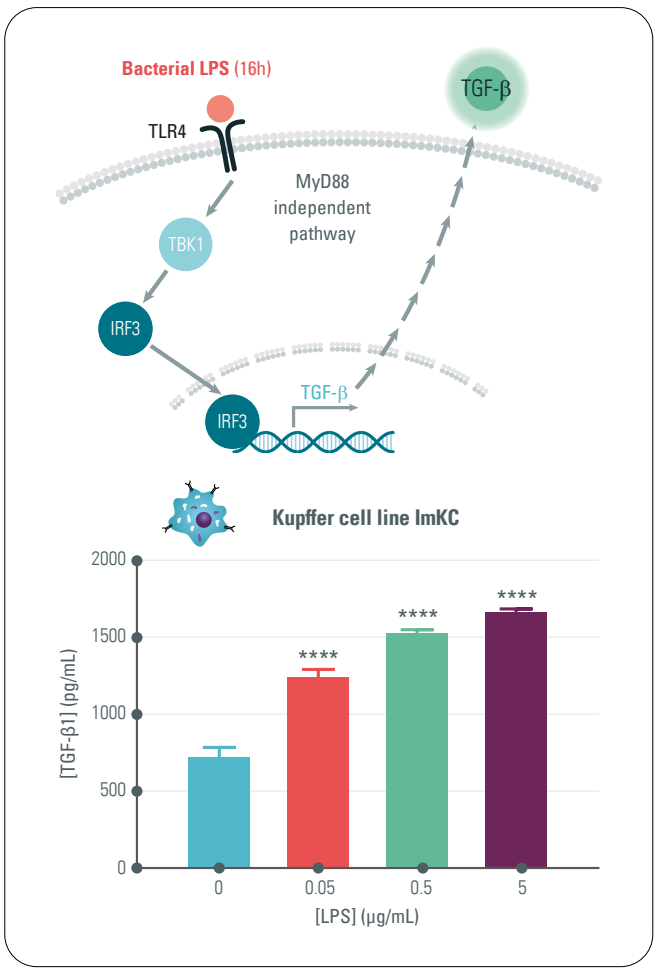


Figure 2: TLR4 signaling and TGF-β release from Kupffer cells activated by LPS

TGF-β/SMAD aignaling activation in hepatocytes & HSCs

The human hepatic cell lines HepG2 and LX-2 (provided by EMD Millipore, part #SCC064) were plated in 96-well culture plates (50,000 and 200,000 cells/well respectively) in complete culture medium and incubated for 24h before treatment with increasing doses of human TGF-β1 for 30 min. After cell lysis, the phosphorylated and total levels of SMAD3 were analyzed in parallel on the same lysates using HTRF SMAD3 phospho-S423/425 and total kits (63ADK025PEG/H and 64ND3PEG/H).

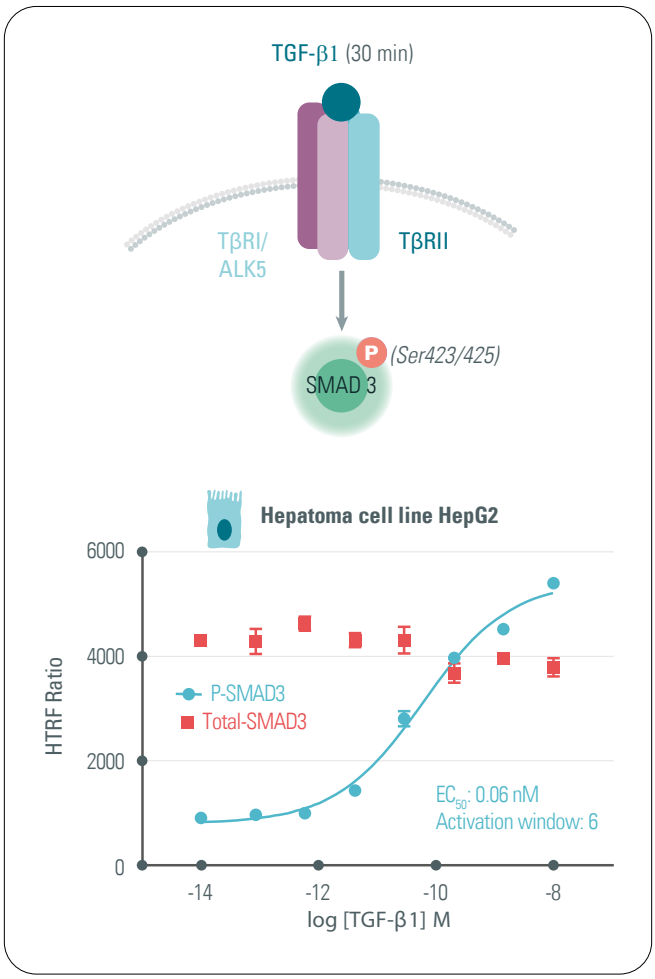


Figure 3: TGF-β/SMAD signaling activation in hepatocytes & HSCS

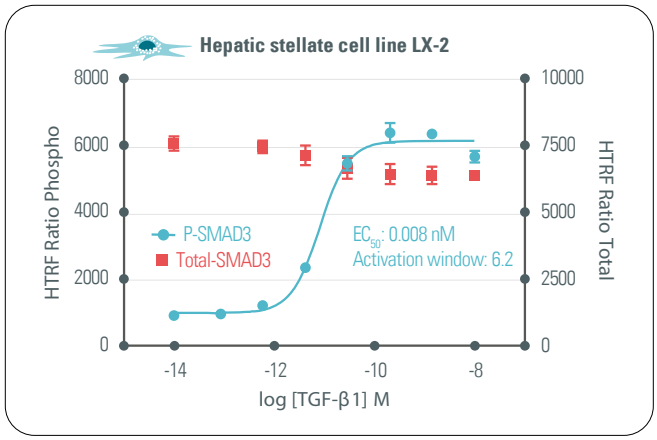


Figure 4: Differentiation of HSCS into myofibroblasts after long-term TGF-β exposure

In both human hepatic cell lines, TGF-β1 induces a 6-fold increase in SMAD3 phosphorylation, which demonstrates the correct activation of TGF-β/SMAD signaling. As expected, the expression level of the protein is not affected by the treatment.

Differentiation of HSCs into myofibroblasts after long-term TGF-β exposure

The LX-2 cell line (provided by EMD Millipore, part #SCC064) was plated in a 96-well culture plate (50,000 cells/well) in complete culture medium and incubated for 24h before treatment with various concentrations of human TGF-β1 for 48h in serum-free and antibiotic-free culture medium supplemented with 0.2% BSA. After cell lysis, the levels of α-SMA were analyzed using the HTRF Alpha-SMA kit (62ASMAPEG/H).

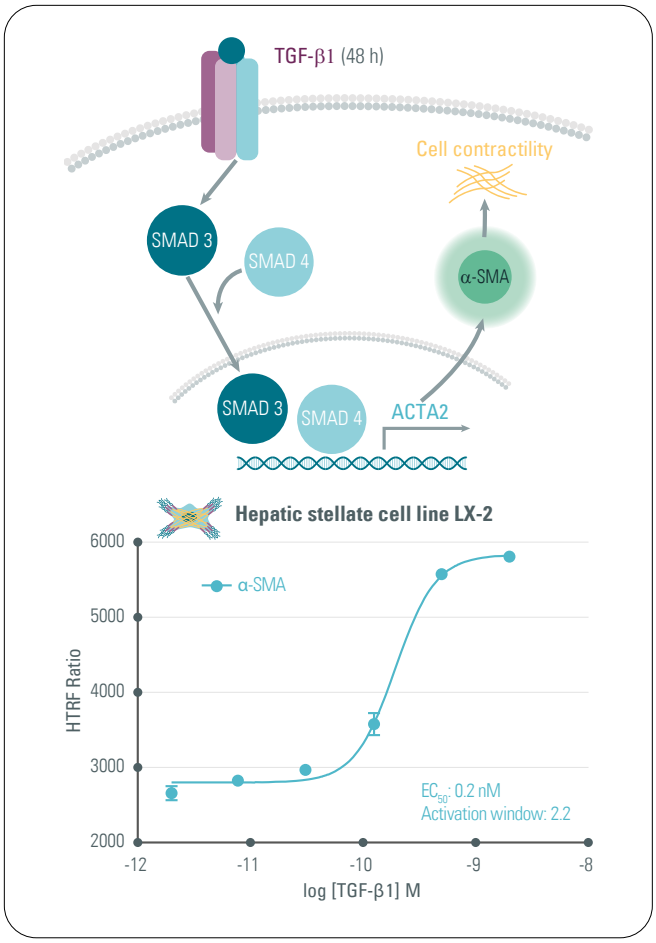


Figure 5: α-SMA detection in LX-2 cell line activated with human TGF-β1

TGF-β1 long-term treatment leads to a two-fold increase in the α-SMA expression level, highlighting the differentiation of HSCs into contractile myofibroblasts responsible for ECM secretion and tissue fibrosis.

Conclusion

This application note explores the modulation of TGF-β1, SMAD3 and α-SMA in various NASH models using the HTRF technology. Taken together, these assays offer a precise insight into the function and dynamics of the liver fibrosis mechanism

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