α-SMA EXPRESSION IN HEPATIC STELLATE CELLS

A comprehensive exploration based on phenotypic and protein observations

APPLICATION NOTE

ABSTRACT α-smooth muscle actin (α-SMA) is a protein that is encoded by the gene ACTA2 in humans, and is a key component of the cellular contractile system. α-SMA expression is considered a reliable marker of hepatic stellate cell (HSC) activation and a key biomarker for liver fibrosis. In the liver, HSCs play a central role in the development of liver fibrosis, transdifferentiating from "quiescent" HSC to a myofibroblastic phenotype in response to Transforming Growth Factor beta (TGF-β), inflammatory processes and ROS.

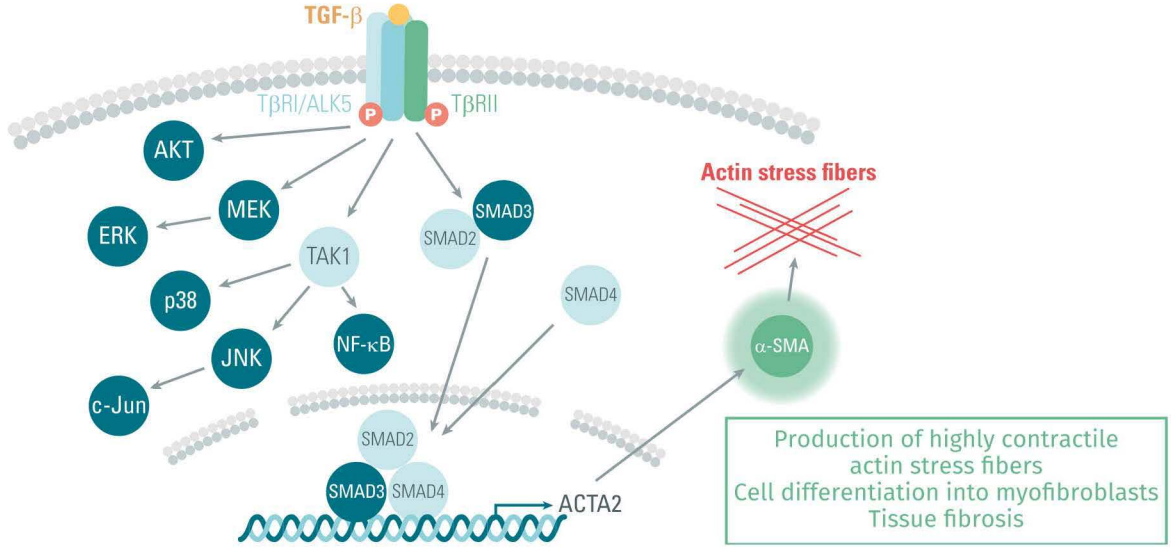
In this application note, the Biotek Cytation™ 5 Cell Imaging Multi-Mode Reader and BioSpa were used to follow the expression of α-SMA in Human LX-2* cells in response to TGF-β stimulation. Phenotypic changes were captured using the instruments' immunofluorescent and live imaging capabilities. Protein expression of α-SMA was confirmed using the HTRF α-SMA kit. These visual and relative observations demonstrate the pivotal role played by α-SMA during myofibroblast differentiation.

THE ROLE OF TGF-β & α-SMA IN FIBROSIS

After tissue injury, TGF-β locally released by inflammatory cells activates resident fibroblasts or quiescent HSCs. This leads to their differentiation into contractile and secretory myofibroblasts, whose role is to migrate into the damaged tissue and synthetize ECM (extracellular matrix) components to repair the wound.

Myofibroblasts are characterized by *de novo* expression of α-SMA, which is incorporated into actin stress fibers and confers a high contractile activity to the cells.

Chronic tissue injury and inflammation lead to persistent *de novo* formation of myofibroblasts (α-SMA+), and excessive deposition of ECM, eventually leading to tissue fibrosis (hepatic, pulmonary, renal, cardiac or dermal). Inhibiting myofibroblast differentiation is therefore a promising therapeutic strategy for the treatment of fibrotic disorders.



*EMD Millipore #SCC064

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MATERIALS, METHODS AND RESULTS

PROCEDURE

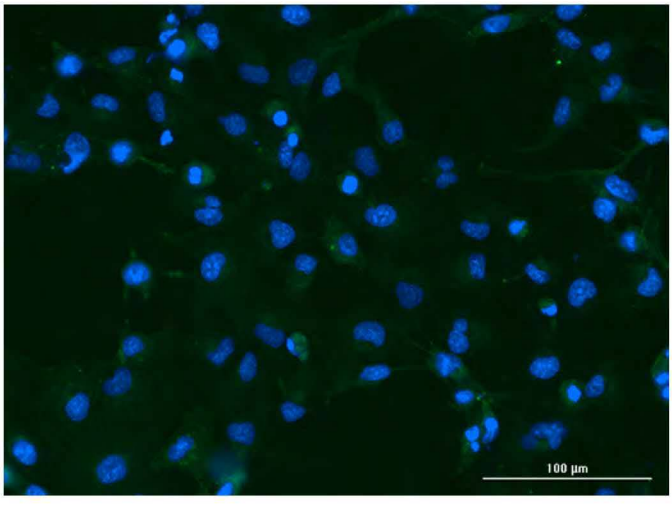
α-smooth muscle actin XP® Rabbit MAb (Alexa Fluor® 488 Conjugate) was purchased from Cell Signaling Technologies (Danvers, MA). Following the TGF-β differentiation protocol described, fixing and staining was carried out following the recommended Cell Signaling Technologies protocol. Briefly, cells were covered in ice-cold 100% methanol and fixed for 15 minutes at -20°C. The methanol was removed, and cells were rinsed three times in 1X PBS for 5 minutes each time. Antibodies were diluted 1/50 in antibody dilution buffer (1X PBS / 1% BSA / 0.3% Triton X-100). 100 mL of diluted antibodies were added to each well. Cells were incubated overnight at 4°C, and then 300 nM (final) DAPI stain were added to each well. After a 10-minute incubation, cells were rinsed 3 times in 1X PBS for 5 minutes each time.

Fixed and stained LX-2 cells were imaged using the Olympus Plan Fluorite 20x objective, and the GFP (469/35 Ex, 525/39 Em) and DAPI (377/50 Ex, 447/60 Em) LED filter cube sets.

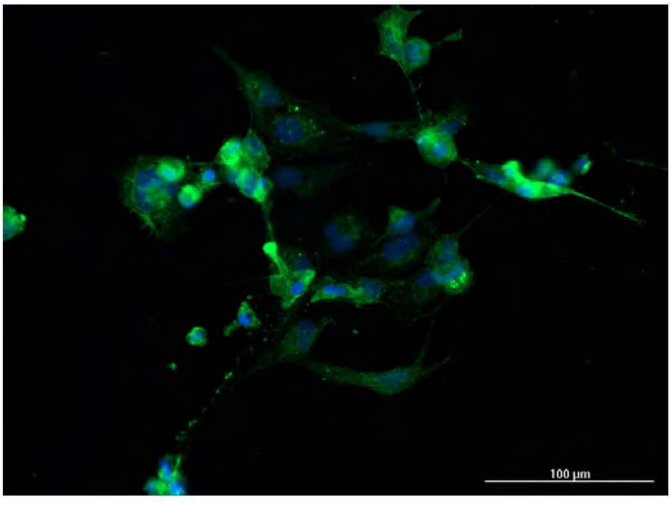
RESULTS

Increased expression of α-SMA in response to TGF- β was captured by immunofluorescent imaging. In response to TGF- β, LX-2* cells transition from a quiescent to a fibroblastic morphotype. The morphological and functional changes include the development of star-shaped structures, the increased production of α-SMA, and the development of cytoskeletal networks, all of which are clearly observable. Cells were co-stained with DAPI to identify the nuclei. These results highlight the role of TGF-β as an important mediator of myofibroblast differentiation.

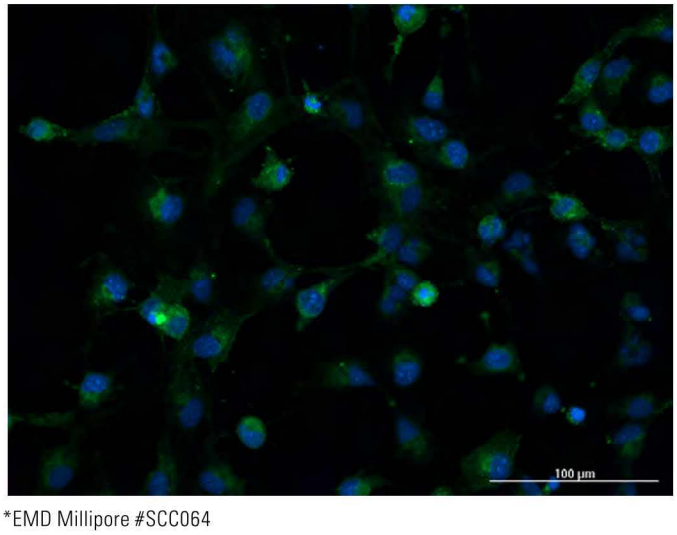
Vehicle alone



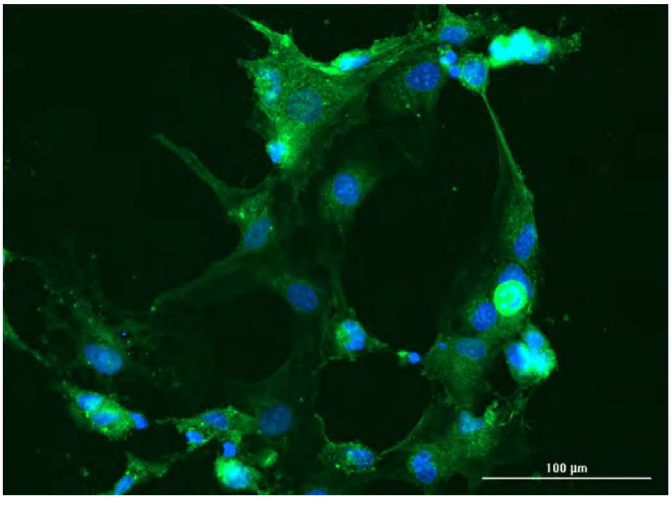
31 pM TGF-β



7.8 pM TGF-β



2 nM TGF-β



*EMD Millipore #SCC064

HTRF α-SMA DETECTION

PROCEDURE

LX-2* cells from EMD Millipore were plated in a culture-treated 96-well plate (Costar 3596) at a density of 12,500 cells per well under 100 µL (culture media: DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (GIBCO, Thermo #10569010), 2% FBS, and 1X Pen/Strep media).

The following day, the cell culture medium was removed, and cells were treated with increasing concentrations of human TGF-β (provided by Cell Signaling Technology #8915) diluted in DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (GIBCO, Thermo #10569010), 0.5% FBS, and 1X Pen/Strep media.

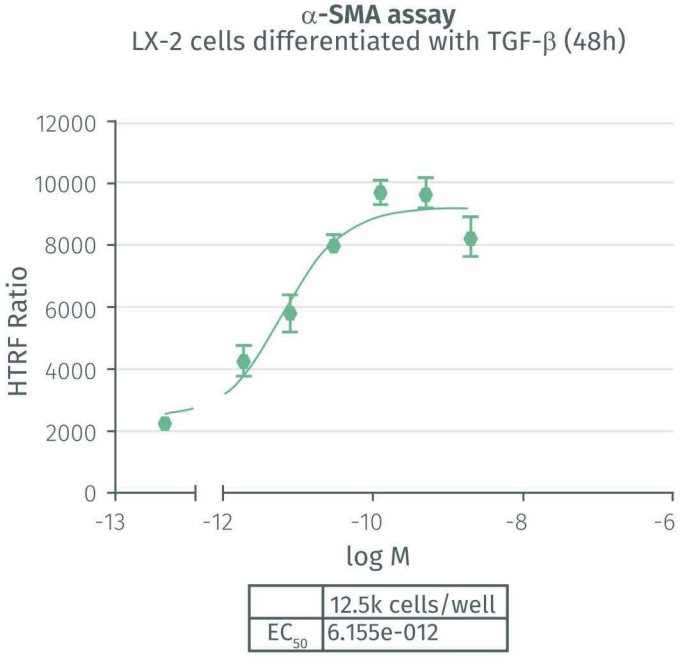
After 48 hours of incubation at 37°C-5% CO2, the medium was removed, and cells were lysed with 50 µL of supplemented lysis buffer #3 (provided in the HTRF® α-SMA kit) for 30 minutes at RT under gentle shaking.

After lysis, 16 µL of lysate were transferred into a low volume white detection microplate and 4 µL of premixed HTRF® α-SMA detection antibodies were added. The HTRF signal was recorded on a Cytation 5 reader after an overnight incubation at RT.

Cisbio Bioassays' α-SMA assay is based on a TR-FRET sandwich immunoassay format comprising two antibodies, one labeled with Eu3+cryptate (donor) and the other with d2 (acceptor). The antibodies specifically bind to α-SMA, and the proximity of donor and acceptor then leads to a fluorescent TR-FRET signal. The protocol is optimized for a 96- or 384-well low volume microplate format (20 µL final), but can easily be further miniaturized or upscaled. Only low sample volumes are needed. The detection reagents may be pre-mixed and added in a single dispensing step for direct detection. No washing is needed at any step.

RESULTS

TGF-β successfully transformed the quiescent hepatic stellate cells LX-2 fibroblasts into α-SMA producing myofibroblasts. TGF-β dose dependently increased the production of α-SMA in LX-2 cells. The potency of the response was measured using the HTRF kit.



	12.5k cells/well
EC_{50}	6.155e-012

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VIDEO RECORDING OF LX-2 CELL DIFFERENTIATION

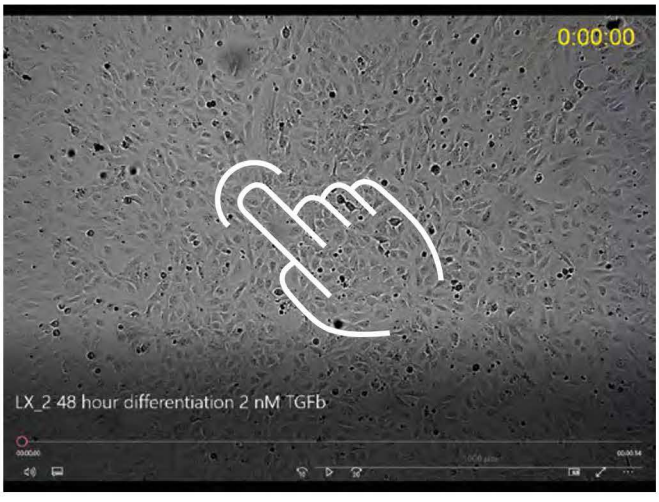
PROCEDURE

The Biotek BioSpa Live Cell Imaging System integrates BioSpa Automated Incubator and Cytation 5 Cell Imaging Multi-Mode Reader to fully automate kinetic live cell imaging and analysis. LX-2 cells were seeded in a Costar 3904 96-well plate at a density of 12,500 cells per well. The following morning, cells were treated with TGF-β and the plate was placed inside the BioSpa 8 Automated Incubator. The BioSpa delivered the plated cells to the Cytation 5 every hour to capture high contrast brightfield images throughout the 48-hour differentiation process, using the 4x Olympus Plan Fluorite objective.

RESULTS

The video in the bottom panel corresponds to the cells addition of 2 nM of TGF-β to a population of cultured LX-2 fibroblasts. The video in the top panel is the control, where LX-2 cells cultured in the same conditions were not exposed to TGF-β. The differentiation into myofibroblasts is clearly observable on the video recording

0 nM TGF-β



2 nM TGF-β

Differentiation of LX-2 fibroblasts into myofibroblasts is observable as early as 24 hours following the addition of TGF-β.

CONCLUSION

In response to TGF-β, the increase of α-SMA protein expression in LX-2 cells can be detected using the immunochemistry capabilities of the Biotek Cytation™ 5 Cell Imaging Multi-Mode Reader. Production of α-SMA can be confirmed using the HTRF homogenous α-SMA assay. These results support the previously described role of α-SMA as a crucial marker of myofibroblast differentiation.

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