

Immunomodulator FTY720 Induces Myofibroblast Differentiation via the Lysophospholipid Receptor S1P₃ and Smad3 Signaling

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The novel immunomodulator FTY720 is an effective immunosuppressive agent in experimental models of transplantation and autoimmunity and is currently undergoing phase III clinical trials for multiple sclerosis. Phosphorylated FTY720 is a structural analogue of sphingosine 1-phosphate (S1P) and therefore acts as a high-affinity agonist at four of the five G protein-coupled S1P receptors. It has been well established that there exists a crosstalk between S1P and transforming growth factor (TGF)- β signaling. Because TGF- β is the most prominent inducer of fibrosis and myofibroblasts are primarily responsible for excessive matrix protein formation, we examined whether FTY720, in analogy to TGF- β , induces differentiation of fibroblasts into myofibroblasts. Indeed, FTY720 provoked myofibroblast differentiation comparable with that of TGF- β . For biological efficacy, FTY720 required endogenous phosphorylation because inhibition of sphingosine kinase completely prevented FTY720 from inducing the differentiation process. Moreover, we identified the lysophospholipid receptor S1P₃ as the crucial receptor subtype for FTY720-induced myofibroblast differentiation because the effect was abolished in fibroblasts isolated from S1P₃ knockout mice. Finally, we determined that downstream of S1P₃ signaling Smad3 activation is essential for myofibroblast differentiation in response to FTY720. Thus, FTY720 may have adverse fibrotic ef-

fects related to its activity on S1P₃ signaling. (Am J Pathol 2007, 170:281–292; DOI: 10.2353/ajpath.2007.060485)

The novel immunomodulator FTY720, which is undergoing phase III clinical trials for the treatment of multiple sclerosis, exhibits a different mechanism of action compared with that of currently used immunosuppressive molecules.^{1–4} The immunomodulator consistently and selectively suppresses the levels of lymphocytes in blood and lymphatic fluid by almost 90% and simultaneously elicits sequestration of recirculating B and T cells into secondary lymphoid organs.² Investigations of the mechanisms of immunosuppression revealed that FTY720 is phosphorylated *in vivo* by sphingosine kinase subtype 2 (SphK2) and that the resulting phosphate ester (FTY720-P) shares striking structural homology to the natural biological mediator sphingosine 1-phosphate (S1P). Thus, FTY720-P is a true agonist of nanomolar potency for the S1P receptors S1P₁, S1P₃, S1P₄, and S1P₅, but not S1P₂.^{3,5,6} Indeed, most actions of FTY720-mediated immunosuppression can be mimicked by non-hydrolyzable phosphonate analogues of FTY720, indicating that the phosphorylated FTY720 is the active principle.⁶ It has been well established that lymphocyte circulation between blood and lymphatic tissue is regulated by the S1P₁ receptor subtype and its natural ligand S1P.³ The importance of this receptor subtype can be shown in mice with a specific deletion of S1P₁ in hematopoietic cells because thymocytes selectively require S1P₁ for egress from the thymus, whereas both T and B cells necessitate this subtype for egress from secondary lymphoid organs.³ Recent studies have indicated that FTY720, after phosphorylation, induces a prolonged

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down-regulation of the receptor subtypes S1P₁ on thymocytes and lymphocytes, depriving them from the obligatory S1P-mediated signal to egress from lymphoid organs.⁷ Besides the action on lymphocyte recirculation, FTY720 maintains vascular integrity of endothelial cells by enhancing adherens junction assembly.⁸

Although sequestration of lymphocytes is solely mediated by S1P₁, unspecific S1P receptor agonists elicit a variety of physiological and pathological responses.⁹ It has been shown that high plasma levels of S1P lead to adverse cardiovascular effects, including hypotension, bradycardia, and coronary artery vasospasm, which are mediated after activation of the receptor subtype S1P₃.¹⁰ Thus, it is not astonishing that high concentrations of FTY720 may also induce S1P₃-mediated bradycardia.⁴

Most recently, we have shown that there also exists a crosstalk between S1P receptors and the signaling cascade of transforming growth factor (TGF)- β in a variety of cells.^{11–13} In general, TGF- β signals through transmembrane receptor serine/threonine kinases to activate signaling intermediates, called Smad proteins, which then translocate into the nucleus and act as transcription factors.¹⁴ In different cell types, it has been shown that S1P augments Smad3 phosphorylation, one of the two homologous proteins, which signals from TGF- β /activin, and that the abrogation of Smad3 prevents S1P-mediated effects, indicating a surprising but essential role of Smad3 in the signaling cascade of the lysophospholipid.^{11,12}

Besides its immunomodulatory effect, TGF- β possesses multiple biological actions, which contribute to the capacity that it plays in many fibrotic diseases.¹⁵ A key role is the TGF- β -mediated differentiation of fibroblasts into myofibroblasts because these cells are primarily responsible for excessive matrix protein formation.¹⁶ Because of a crosstalk between S1P and TGF- β signaling, it was therefore of interest whether FTY720 may also influence myofibroblast differentiation. Indeed, here we show that FTY720 strongly increases myofibroblast differentiation as a result of interaction of the phosphorylated analogue with the S1P₃ receptor subtype and the subsequent activation of Smad3.

Materials and Methods

Materials

N,N-Dimethylsphingosine (DMS), pertussis toxin (PTX), and protein G agarose were purchased from Calbiochem (Bad Soden, Germany). FuGene was from Roche Diagnostics (Mannheim, Germany). Mouse monoclonal anti-Smad1,2,3, rabbit polyclonal anti-S1P₁, goat polyclonal anti-S1P₃, goat polyclonal anti-S1P₄, goat polyclonal anti-S1P₅ antibodies, goat polyclonal anti-TGF- β antibodies, normal goat IgG, and anti-goat IgG-horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). LumiGlo reagent, peroxide, and anti-rabbit and anti-mouse IgG horseradish peroxidase were obtained from New England Biolabs (Beverly, MA). FTY720 was purchased from Calbiochem. Bovine serum

albumin, fetal bovine serum (FBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and L -glutamine solution were from Seromed Biochrom (Berlin, Germany). Aprotinin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide, Dulbecco's modified Eagle's medium (DMEM), ethylenediaminetetraacetic acid (EDTA), leupeptin, Mowiol, murine monoclonal anti- α -smooth muscle actin (α -SMA) antibodies, fluorescein isothiocyanate (FITC)-linked anti-mouse antibodies, penicillin, pepstatin, sodium dodecyl sulfate (SDS), TGF- β 1, sodium fluoride, sodium orthovanadate, streptomycin, Tris, Triton X-100, trypsin, and Tween 20 were purchased from Sigma (Deisenhofen, Germany). OptiMEM was from Invitrogen (Karlsruhe, Germany). Oligonucleotides were synthesized at Tib Molbiol (Berlin, Germany). Polyvinylidene difluoride membranes were purchased from Millipore (Schwalbach, Germany).

Synthesis of FTY720-P

FTY720-P (phosphoric acid mono[(*R/S*)-2-amino-2-hydroxymethyl-4-(4-octylphenyl)butyl]ester) was synthesized from FTY720 (2-amino-2-(2-[4-octylphenyl]ethyl)-1,3-propanediol) as recently described.¹⁷ In brief, FTY720 was protected as an oxazolidinone by the addition of benzyl chloroformate. Then, phosphorylation of the free hydroxyl group was performed by the addition of 3-(diethylamino)-1,5-dihydro-2,4,3-benzodioxaphosphetriphenyl phosphite and a subsequent oxidation to obtain (*R/S*)-4-[2-(4-octylphenyl)ethyl]-4-(3-oxo-1,5-dihydro-3 λ^5 -benzo[e][1,3,2]dioxaphosphepin-3-yloxymethyl)oxazolidin-2-one. The phosphate-protecting group was removed by hydrogenation, and the oxazolidinone was cleaved with lithium hydroxide yielding to FTY720-P. Identity was checked by electrospray ionization time-of-flight mass spectrometry using an Agilent 6210 TOF LC/MS (Waldbronn, Germany). Electrospray ionization-mass spectrometry, *m/z*: 386 ($M - H$)⁻. Moreover, purity of FTY720-P was measured by high-performance liquid chromatography (HPLC).¹⁸ Therefore, synthesized FTY720-P or standard FTY720 was dissolved in 275 μl of methanol/0.07 mol/L K₂HPO₄ (9:1). A derivatization mixture of 10 mg of *o*-phthalaldehyde, 200 μl of ethanol, 10 μl of 2-mercaptoethanol, and 10 ml of 3% boric acid was prepared and adjusted to pH 10.5 with KOH. Twenty-five μl of the derivatization mixture was added to the resolved FTY720 or FTY720-P for 15 minutes at room temperature. The derivatives were analyzed by a Merck Hitachi LaChrom HPLC system (Merck Hitachi, Darmstadt, Germany) using a RP 18 Kromasil column (Chromatographie Service, Langerwehe, Germany). Separation was done with a gradient of methanol and 0.07 mol/L K₂HPO₄. Resulting profiles were evaluated using the Merck system manager software, indicating no detectable amount of FTY720 in the synthesized FTY720-P.

Preparation of Human Fibroblasts

To isolate human fibroblasts, juvenile foreskin from surgery was incubated at 37°C for 2.5 hours in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinization was terminated by the addition of DMEM containing 10% FBS. Cells were washed with phosphate-buffered saline (PBS) and centrifuged at 250 × *g* for 5 minutes. The pellet was resuspended in DMEM containing 7.5% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Fibroblasts were pooled from several donors and cultured at 37°C in 5% CO₂. Only cells of the third to sixth passage were used for the experiments.

Preparation of Wild-Type and Smad3^(-/-) Fibroblasts

Murine fibroblasts, isolated from polymerase chain reaction-genotyped wild-type and Smad3 knockout newborn mice, were kindly provided from Dr. Anita Roberts (National Institutes of Health, National Cancer Institute, Bethesda, MD).¹⁹ Cells were cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. For detection of wild-type and Smad3^(-/-) fibroblasts the primer sequences 5'-CCACTTCATTGCCATATGCCCTG-3' (located 5' to the deletion) and 5'-CCCGAACAGTTGGATTCACACA-3' (located within the deletion) were used. Smad3^(-/-) fibroblasts were identified using the primer located to the 5' deletion and a primer specific for the pLoxpneo cassette (5'-CCAGACTGCCTTGGGAAAA-GC-3').

Preparation of Wild-Type and S1P₃^(-/-) Fibroblasts

Wild-type and S1P₃ knockout mice were generated by Dr. Jerold Chun as recently described.²⁰ To isolate murine fibroblasts, skin was incubated at 37°C for 2.5 hours in a solution of 0.25% trypsin and 0.2% EDTA. Trypsinization was completed by the addition of DMEM containing 10% FBS. Isolated fibroblasts were washed with PBS and centrifuged at 250 × *g* for 5 minutes. The pellet was resuspended in DMEM containing 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and cultured at 37°C in 5% CO₂. Cells were genotyped by polymerase chain reaction. The following primers were used: 5'-CACAGCAAGCAGACCTCCAGA-3', 5'-TGGTGTGCGGCTGTCTAGTCAA-3', and 5'-ATCG-ATACCGTCGATCGACCT-3'.

Real-Time Polymerase Chain Reaction (PCR)

Real-time PCR assays were performed using the SYBR Green PCR Master Mix on ABI Prism 7900HT sequence detection system according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Amplification was performed in 10- μ l reactions (primer concentration, 250 nmol/L, 1× SYBR Green Master Mix) containing 2 μ l

of cDNA (equivalent to 10 ng of total RNA) in 40 cycles of 95°C, 15 seconds, 60°C, 1 minute. Primers were purchased at SuperArray Bioscience Corporation (Frederick, MD). Total RNA of three different sets of fibroblasts were used to analyze receptor expression. Data normalization was performed using GAPDH as reference gene. Relative mRNA expression was quantified using the comparative C_T method according to the ABI manual.

Cell Viability Assay

Cell viability was measured by the MTT dye reduction assay. Cells, seeded into 24-well plates for 24 hours, were incubated with FTY720 or FTY720-P for 24 hours at 37°C in 5% CO₂. After the addition of 100 μ l of MTT solution (5 mg/ml) per well, the plates were incubated for another 4 hours. The supernatants were removed, and the formazan crystals were solubilized in 1 ml of dimethyl sulfoxide. The optical density was determined at 540 nm using a scanning microplate spectrophotometer (Multiscan Plus; Labsystems, Helsinki, Finland).

Immunofluorescence Microscopy of α -SMA

Fibroblasts were seeded into 12-well plates, each containing a glass coverslip, and cultured for 24 hours in DMEM containing 2 mmol/L L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 7.5% FBS. Then they were serum-deprived for 48 hours in DMEM supplemented with 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Quiescent fibroblasts were stimulated with the indicated substances for 72 hours. Cells were washed with ice-cold PBS and fixed in methanol at 4°C for 2 minutes. Cells were treated with blocking buffer (1% bovine serum albumin in PBS) for 30 minutes followed by incubation with murine anti- α -SMA antibodies (1:50 diluted in blocking buffer). Coverslips were washed three times with blocking buffer and incubated with FITC-linked anti-mouse antibodies (1:125 diluted in blocking buffer). After 30 minutes, fibroblasts were washed three times with blocking buffer and fixed by a Mowiol mounting medium. Staining was examined using the Olympus BX41 fluorescence microscope (Hamburg, Germany) and documented by the digital camera Nikon DXM1200 (Düsseldorf, Germany). Appropriate emission filter settings and controls were included for bleed-through effects.

Immunoprecipitation

Human fibroblasts were seeded in six-well plates and cultured for 24 hours in DMEM containing 7.5% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin, and then medium was replaced by HEPES buffer (1 mol/L) for 2 hours. Cells were treated with TGF- β (2 ng/ml) or FTY720 (1 μ mol/L) for 30 minutes. Fibroblasts were rinsed twice with ice-cold PBS and harvested in radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS), containing pro-

tease inhibitors (1 mmol/L phenylmethyl sulfonyl fluoride, 1 mmol/L EDTA, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 μ g/ml pepstatin) and phosphatase inhibitors (1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, and 40 mmol/L β -glycerophosphate). Lysates were centrifuged at 14,000 $\times g$ for 30 minutes. One hundred μ g of lysate protein was immunoprecipitated overnight at 4°C with 0.2 μ g of anti-Smad1,2,3 antibodies or 0.2 μ g of normal goat IgG (IgG control), followed by a precipitation with 10 μ l of protein G plus agarose at 4°C for 90 minutes. After four washes with complete RIPA buffer, the immunoprecipitates were eluted by boiling for 5 minutes in 60 μ l of SDS sample buffer (100 mmol/L Tris/HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol, and 200 mmol/L dithiothreitol).

Immunoblotting

For Western blot analysis, immunoprecipitates (20 μ l) or cell lysates (10 to 20 μ g of protein) were separated by SDS/polyacrylamide gel electrophoresis. Gels were blotted overnight onto polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline (TBS)-Tween 20 (0.1%) overnight at 4°C, membranes were incubated with the indicated specific primary antibodies for 0.5 or 2 hours at room temperature. The blots were washed three times in TBS-Tween 20 followed by incubation with the secondary horseradish peroxidase-conjugated antibodies for 1 hour at room temperature. Immunocomplexes were detected using an enhanced chemiluminescence detection method. Densitometry of films was performed using the Syngene GeneGenius (Cambridge, UK).

Anti-Sense Oligonucleotides

Anti-sense oligonucleotides (ASOs) were designed to surround the translational initiation site, a place empirically known to be most effective for inhibition of gene expression. The following specific ASOs as well as same-length control oligonucleotides (with the same nucleotides but randomly scrambled sequence) were synthesized: Smad3 ASO: 5'-GCAGGATGGACGACAT-3', control oligonucleotides 5'-GTGGACAGCTAGAGAC-3'; SphK2 ASO: 5'-CAGGGGAAGAGGCAGGTCAGACA-3', control oligonucleotides: 5'-TGCAAGCTCAACACCCACATA-3'; S1P₁: ASO 5'-GACGCTGGTGGGCCCCAT-3', control oligonucleotides: 5'-ATGGGGCCACCAGC-GTC-3'; S1P₃: ASO 5'-CGGGAGGGCAGTTGCCAT-3', control oligonucleotides: 5'-ATGGCAACTGCCCTCCCG-3'; S1P₄: ASO 5'-GAAGGCCAGCAGGATCATCCAC-3', control oligonucleotides: 5'-ACCTAGCCACACCCTCC-ATGAAGGC-3'; S1P₅: ASO 5'-CAACATGCCACAAAGGCCAGGAG-3', control oligonucleotides: 5'-GCAACAACATAACGGCCAGCAG-3'. Cells were seeded in six-well plates and cultured in DMEM supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin for 12 hours. Control oligonucleotides and ASOs were solubilized in OptiMEM and FuGENE (1 μ g of DNA/2 μ l) to achieve a final concentration

of 500 nmol/L of oligonucleotides. Then the solution was added to fibroblasts for 72 hours. Abrogation of protein expression was verified by immunoblotting.

Measurement of TGF- β Secretion

Fibroblasts (1 $\times 10^5$ cells/ml) were stimulated with FTY720 (1 μ mol/L) throughout a time period of 24 hours. Then levels of TGF- β in the supernatant were quantified by selective enzyme-linked immunosorbent assay kits following the instructions of the manufacturer (Amersham Pharmacia Biotech, Freiburg, Germany). For measurement of latent complexes of TGF- β , activation was accomplished by acid treatment. Therefore, 0.5 ml of cell culture supernatants were treated with 0.1 ml of 1 mol/L HCl, incubated for 10 minutes, and then neutralized with 0.1 ml of 1.2 mol/L NaOH/0.5 mol/L HEPES. Samples were analyzed by a Fluostar Optima ELISA reader from BMG Labtech (Offenburg, Germany). The detection limit was 4 pg/ml.

Results

FTY720 Induces Transformation of Fibroblasts into Myofibroblasts

The expression of α -SMA is one of the most prominent features of myofibroblasts, which have a phenotype intermediate between smooth muscle cells and fibroblasts. TGF- β has been indicated as the crucial cytokine to induce transformation of fibroblasts into myofibroblasts.¹⁶ Most recently, we figured out that there exists a crosstalk between TGF- β and S1P receptors and that S1P mimics biological effects of TGF- β in dendritic cells.¹¹ Based on the structural similarity between S1P and the phosphorylated FTY720, we investigated whether the immunomodulator FTY720 might also influence myofibroblast differentiation. Therefore, α -SMA expression in response to FTY720 was measured by Western blotting and immunofluorescence microscopy in primary human fibroblasts. For positive control experiments, fibroblasts were treated with TGF- β , and immunofluorescence indicated a pronounced α -SMA formation confirming that TGF- β stimulates the fibroblasts to differentiate into myofibroblasts (Figure 1). Most interestingly, treatment of primary cells with FTY720 also resulted in a distinct expression of α -SMA (Figure 1). Immunofluorescence of α -SMA in response to FTY720 showed a distinct appearance of numerous bundles of actin microfilaments comparable with TGF- β , whereas the cell shape was slightly elongated. The number of fibroblasts expressing α -SMA was drastically increased in a dose-dependent manner (Figure 1). A significant increase was detected at a concentration of 0.1 μ mol/L FTY720, whereas a maximal effect occurred at 1 μ mol/L inducing a similarly strong expression of α -SMA as the most effective dose of TGF- β (2 ng/ml) (Figure 1). It should be noted that higher concentrations of FTY720 did not further increase α -SMA formation attributable to a toxic

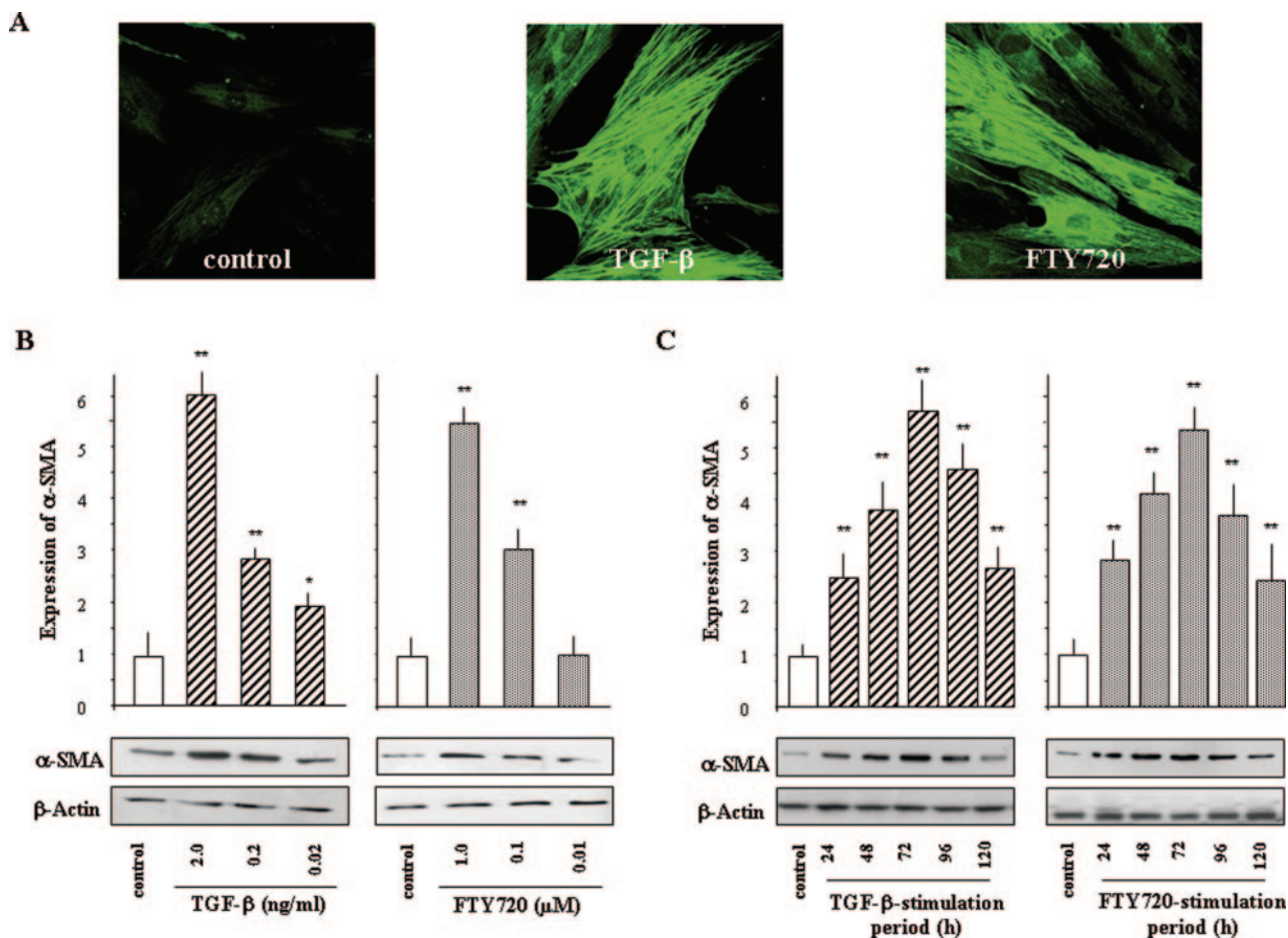


Figure 1. TGF- β and FTY720 induce myofibroblast differentiation. **A:** Human fibroblasts were stimulated with TGF- β (2 ng/ml) or FTY720 (1 μ mol/L) for 72 hours followed by an immunofluorescence analysis of α -SMA. **B and C:** Cells were treated with the indicated concentrations of TGF- β or FTY720 for 72 hours (**B**) or with 2 ng/ml TGF- β or 1 μ mol/L FTY720 for different stimulation periods (**C**). Then α -SMA was measured by Western blot analysis as described in Materials and Methods. All results were confirmed in three independent experiments. Densitometric analysis of α -SMA formation was performed after Western blot analysis. Values are normalized to β -actin levels and are expressed as an \times -fold increase of α -SMA formation compared with untreated cells \pm SEM from at least three experiments. * P < 0.05 and ** P < 0.001 indicate a statistically significant difference versus unstimulated control cells. Original magnifications, \times 400.

effect of the immunosuppressive agent (data not shown). Furthermore, in analogy to TGF- β , a significant increase of α -SMA was first detected after a 24-hour treatment of fibroblasts with FTY720, whereas a maximal response was visible after 72 hours (Figure 1).

Phosphorylation of FTY720 Is Required for Myofibroblast Differentiation

Although FTY720 induces myofibroblast differentiation, a variety of studies indicate that phosphorylation of FTY720 by sphingosine kinase is necessary for its biological effects.^{5,6} Therefore, we measured whether FTY720-P is also able to differentiate fibroblasts into myofibroblasts. Indeed, in Figure 2 it is presented that the phosphorylated product enhanced α -SMA formation comparable with FTY720. It is of interest that the kinetic of α -SMA formation induced by FTY720-P was very similar to FTY720 showing a maximal effect after an incubation period of 72 to 96 hours (Figure 2). These results suggest that fibroblasts induce a rapid

phosphorylation of FTY720. Because FTY720-P shares structural homology to the natural biological mediator S1P, we examined whether the lysophospholipid S1P also induces myofibroblast differentiation. Actually, in Figure 2 it is shown that treatment of primary cells with S1P also resulted in the formation of α -SMA. A maximal effect occurred after an in-cubation period of 72 hours with 10 μ mol/L S1P (Figure 2).

To examine whether the action of FTY720 is mediated by the phosphorylated product, conversion of FTY720 to FTY720-P was blocked by the sphingosine kinase inhibitor DMS. Indeed, when fibroblasts were treated with 5 μ mol/L DMS, the ability of FTY720 to enhance α -SMA was almost completely diminished, whereas TGF- β -induced α -SMA formation was not affected in the presence of DMS (Figure 3, A and B). Moreover, several studies indicate that SphK2 is mainly responsible for the phosphorylation of FTY720. Therefore, we measured the ability of FTY720 to induce α -SMA expression after treatment with SphK2-ASO. In Figure 3C, it is shown that control oligonucleotides did not reduce α -SMA formation in response to FTY720. But when cells were treated with SphK2-ASO,

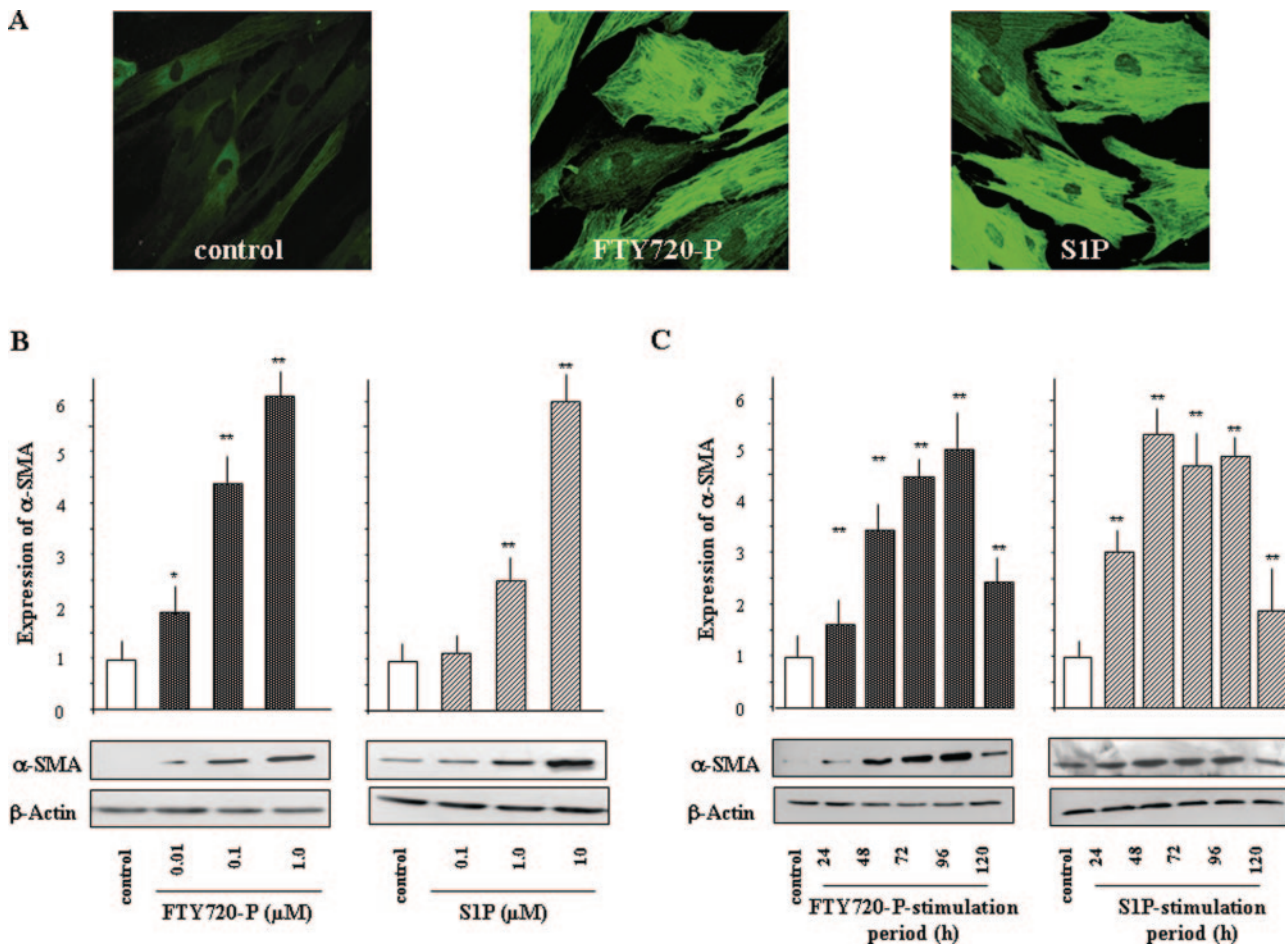


Figure 2. The phosphorylated compound FTY720-P and S1P induce myofibroblast differentiation. **A:** Human fibroblasts were stimulated with FTY720-P (1 μmol/L) or S1P (10 μmol/L) for 72 hours followed by an immunofluorescence analysis of α-SMA. **B and C:** Cells were treated with the indicated concentrations of FTY720-P or S1P for 72 hours (**B**) or with 1 μmol/L FTY720-P or 10 μmol/L S1P for different time periods (**C**). α-SMA was measured by Western blot analysis, and its quantitative determination by densitometry normalized to β-actin levels. All results were confirmed in three independent experiments. **P* < 0.05, ***P* < 0.001. Original magnifications, ×400.

FTY720 but not TGF-β lost the capability to induce expression of α-SMA. These results clearly indicate that FTY720-P is the active metabolite to induce transformation of fibroblasts.

FTY720-P Mediates Myofibroblast Differentiation through Activation of the S1P₃ Receptor

Because FTY720-P is a potent agonist of four of the five G protein-coupled S1P receptors, we performed real-time PCR to quantify mRNA of S1P receptors in primary fibroblasts. In agreement with further studies, mRNA of all five S1P receptor subtypes were expressed in fibroblasts.²¹ The relative amount of S1P receptor mRNA was S1P₃ >> S1P₁ > S1P₂ > S1P₅ > S1P₄ (Figure 4B). To prove the involvement of Gα_i-sensitive S1P receptors in the FTY720-mediated myofibroblast differentiation, we measured the FTY720-induced α-SMA formation in the presence of PTX. Preincubation of fibroblasts with PTX almost completely abolished the ability of FTY720 to induce α-SMA expression, indicating that it is mainly dependent

on S1P receptors interacting with Gα_i (Figure 4). It should be stated that PTX did not influence the aptitude of TGF-β to induce α-SMA expression (Figure 4). To further elucidate which of the S1P receptor subtypes are essential for myofibroblast formation in response to FTY720, expression of S1P receptors were abrogated by the use of ASO. Treatment of cells with S1P₁-ASO, S1P₃-ASO, S1P₄-ASO, and S1P₅-ASO resulted in a serious reduction of protein levels (Figure 5A). Most interestingly, measurement of α-SMA expression revealed that S1P₃ is critical for the FTY720-mediated myofibroblast differentiation. Thus, abrogation of S1P₃ significantly inhibited the capability of FTY720 to increase levels of α-SMA, whereas abrogation of S1P₁, S1P₄, and S1P₅ did not significantly decrease α-SMA expression in response to FTY720 (Figure 5B). To more rigorously confirm that S1P₃ is essential for FTY720-mediated myofibroblast differentiation, we used primary fibroblasts isolated from wild-type or S1P₃ knockout mice. In analogy to our results in primary human fibroblasts, FTY720 was able to enhance the expression of α-SMA in control fibroblasts derived from wild-type mice. On the contrary, S1P₃-deficient fibroblasts did not augment lev-

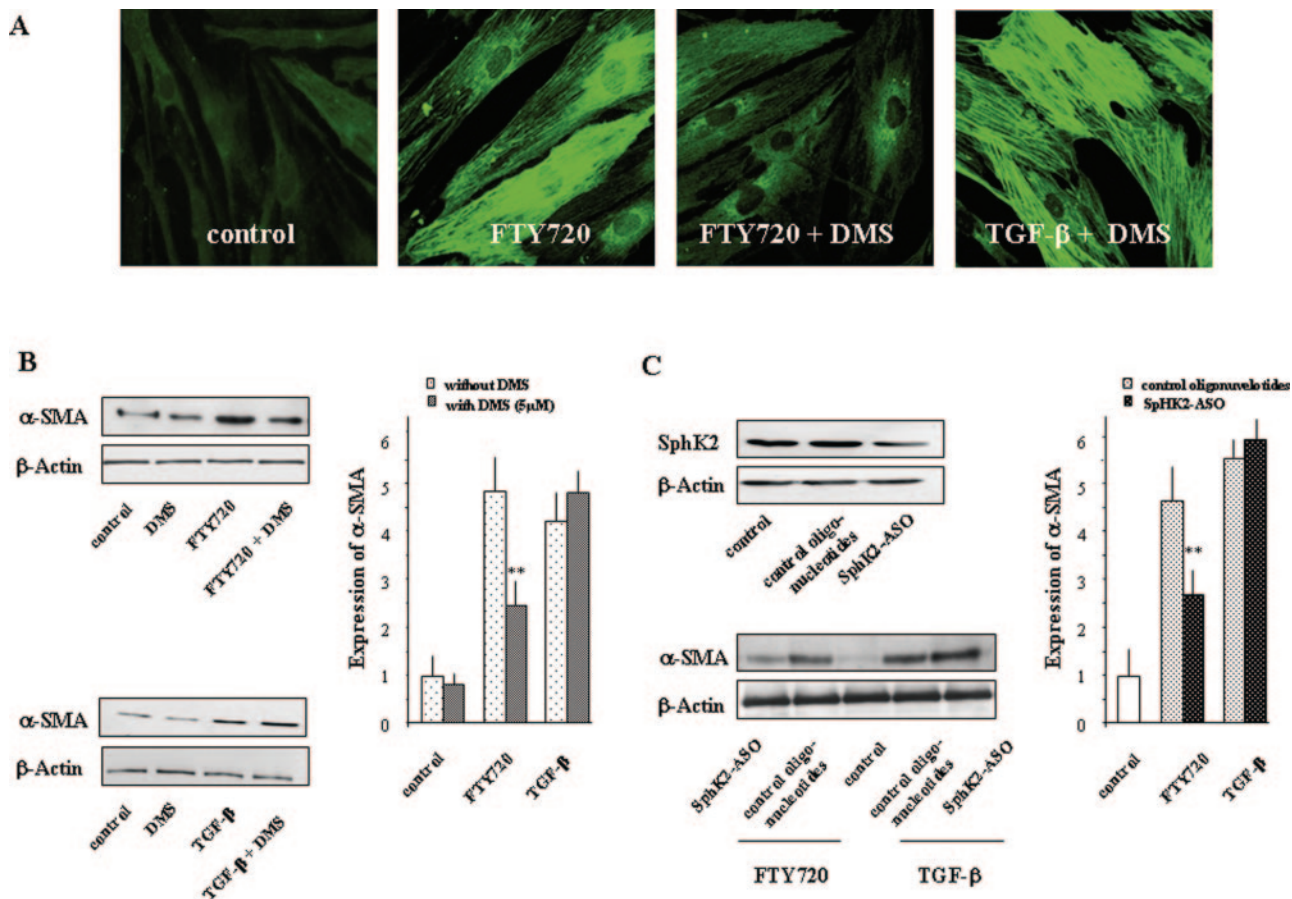


Figure 3. The phosphorylated metabolite FTY720-P is responsible for FTY720-mediated myofibroblast differentiation. **A** and **B**: Human fibroblasts were stimulated with FTY720 (1 μmol/L) or TGF-β (2 ng/ml) for 72 hours in the presence of DMS (5 μmol/L) followed by an immunofluorescence analysis (**A**) or Western blot analysis and densitometric quantification of α-SMA normalized to β-actin (**B**). Cells were transfected with SphK2-ASO (500 nmol/L) or control oligonucleotides (500 nmol/L) for 72 hours. **C**: Western blot analysis confirmed the relative abundance of the SphK2 protein expressed after SphK2-ASO pretreatment. Then cells were treated with FTY720 (1 μmol/L) or TGF-β (2 ng/ml) for 72 hours, and α-SMA was measured by Western blot analysis and densitometric quantification of α-SMA normalized to β-actin. All results were confirmed in three independent experiments. ***P* < 0.001. Original magnifications, ×400.

els of α-SMA in response to FTY720, whereas TGF-β was able to induce α-SMA expression in both wild-type and S1P₃-deficient fibroblasts (Figure 6).

FTY720 Mediates Myofibroblast Differentiation through Activation of Smad3 Protein

In several studies it has been demonstrated that activation of Smad3 is critical for TGF-β-induced transformation of fibroblasts into myofibroblasts. Because we found that TGF-β and S1P activated Smad3 in XS52 dendritic cells,¹¹ it was of fundamental interest to determine whether FTY720 is also able to activate Smad signaling in primary fibroblasts. To address this, FTY720- or TGF-β-stimulated cells were immunoprecipitated with anti-Smad1,2,3 and immunoblotted with anti-Smad4 antibodies. Indeed, Smad4 was co-immunoprecipitated after treatment with both, FTY720 and TGF-β, indicating an activation of the Smad cascade in response to both stimuli (Figure 7B). To further explore a role of Smad3 in FTY720-mediated differentiation of fibroblasts, α-SMA expression was measured after treatment with Smad3-ASO, which significantly reduced levels of Smad3 proteins

(Figure 7C). Most interestingly, FTY720-mediated α-SMA formation was significantly diminished in the presence of Smad3-ASO compared with a treatment with control oligonucleotides (Figure 7D). To more rigorously substantiate that these effects of FTY720 were dependent on Smad3, we used Smad3 knockout and wild-type fibroblasts. Similar to our ASO experiments, a strong α-SMA expression was only visible in wild-type fibroblasts after treatment with FTY720 or TGF-β as positive control. On the contrary, Smad3-deficient fibroblasts did not induce formation of α-SMA in response to both stimulants (Figure 7E).

To examine whether FTY720-mediated differentiation into myofibroblast is a consequence of TGF-β secretion, we measured TGF-β levels after treatment with FTY720. Indeed, basal levels of TGF-β (5.3 pg/ml) or latent complexes of TGF-β (83.5 pg/ml) were not increased throughout a 24-hour stimulation period with FTY720 (4.5 or 78.7 pg/ml, respectively). To further substantiate that FTY720-induced myofibroblast differentiation is independent of TGF-β release, α-SMA formation was visualized under immunoneutralized conditions. As presented in Figure 7A, FTY720 induced α-SMA expression in the presence

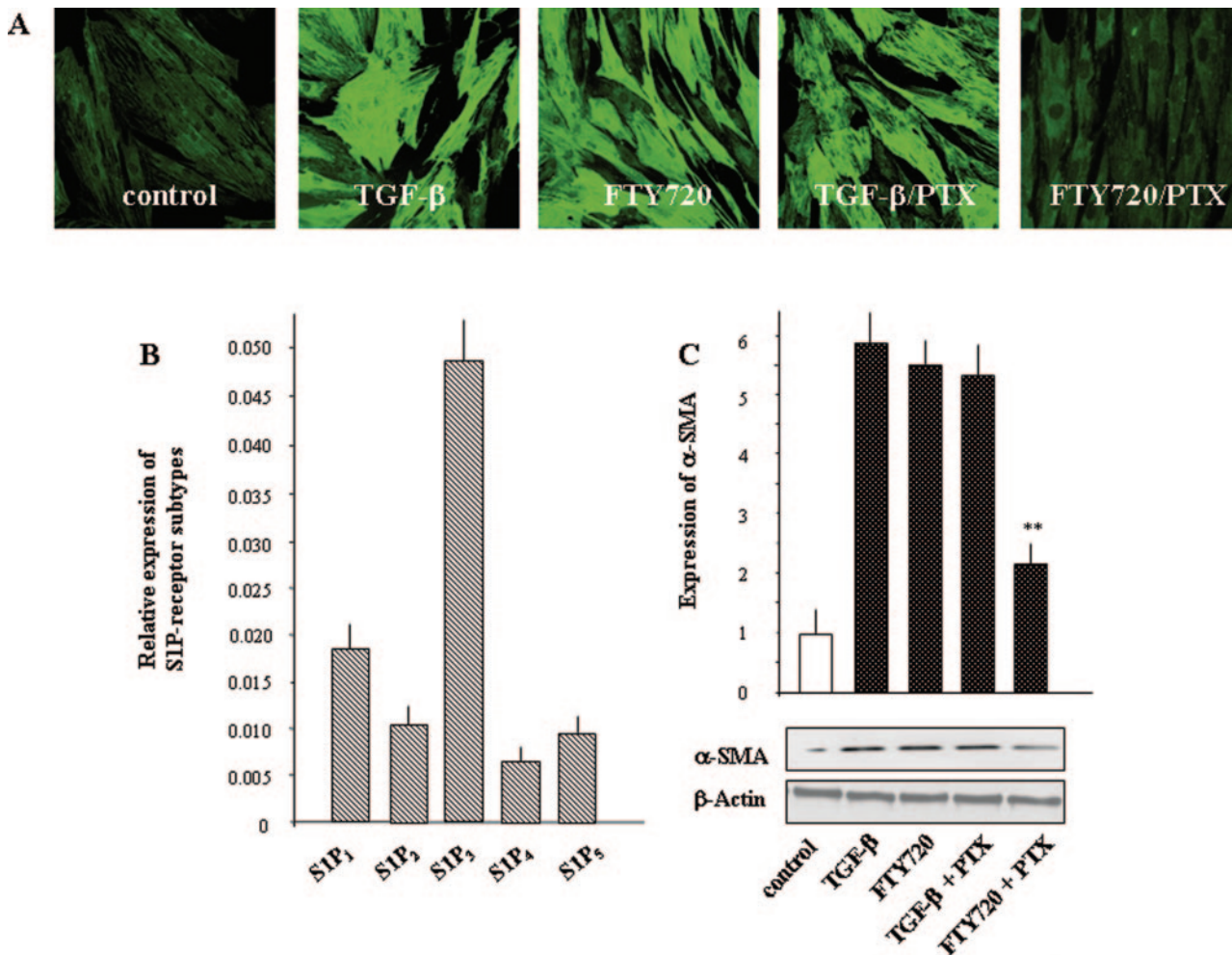


Figure 4. Involvement of $G\alpha_i$ protein-coupled receptors in FTY720-mediated myofibroblast differentiation. **B:** Real-time PCR was performed as described in Materials and Methods, indicating the presence of mRNA of all five S1P receptor subtypes. Human fibroblasts were pretreated with PTX (200 ng/ml) for 3 hours and then stimulated with TGF- β (2 ng/ml) or FTY720 (1 μ mol/L) for 72 hours. **A** and **C:** α -SMA was determined by immunofluorescence (**A**) or Western blot analysis and its densitometric quantification normalized to β -actin (**C**). All results were confirmed in three independent experiments. ** $P < 0.001$. Original magnifications, $\times 200$.

of anti-TGF- β antibodies, whereas the effect in response to TGF- β was completely diminished. These results clearly indicate that FTY720 induces differentiation of fibroblasts into myofibroblasts via activation of S1P₃. Although FTY720 does not induce TGF- β secretion, Smad3 is crucial for the differentiation-inducing effect.

Discussion

FTY720 has been discovered as an immunomodulator with a novel mode of action that is not similar to that of classical immunosuppressive drugs.² It has been shown that FTY720 is rapidly phosphorylated *in vivo* and that FTY720-P is the biologically active principle. This compound shares striking structural homology with S1P, thus acting as a high-affinity agonist at four of the five S1P receptor subtypes.^{3,5,6,22–24}

It is of interest that FTY720-P does not only influence immunomodulation by internalization of S1P₁ on thymocytes and lymphocytes, but also possesses a variety of further biological actions via stimulation of different S1P

receptor subtypes. Both S1P and FTY720-P have been indicated to interact with the endothelium resulting in the regulation of the vascular permeability.^{25,26} Moreover, S1P and FTY720 stimulate endothelial nitric oxide synthase (eNOS) inducing a nitric oxide (NO)-dependent vasorelaxation of precontracted isolated arteries via the receptor subtype S1P₃.^{27,28} Because eNOS stimulation is associated with a cytoprotective role in the development of several cardiovascular diseases, FTY720 may also possess additional therapeutic effects that are independent of an immunomodulating role.²⁷ But a reduction of the heart rate has also been reported in response to FTY720, limiting its therapeutic feasibility.⁴ This effect seems to be mediated by an activation of S1P₃ because this receptor subtype is dominantly expressed in the heart and moreover FTY720 loses its ability to reduce the heart rate in S1P₃-deficient mice.^{10,29} It is of interest that the FTY720-mediated bradycardia could be reversed in the presence of β 2 receptor agonists or atropine indicating a cross-communication between S1P and muscarinic receptors.⁴ This is in agreement with several studies

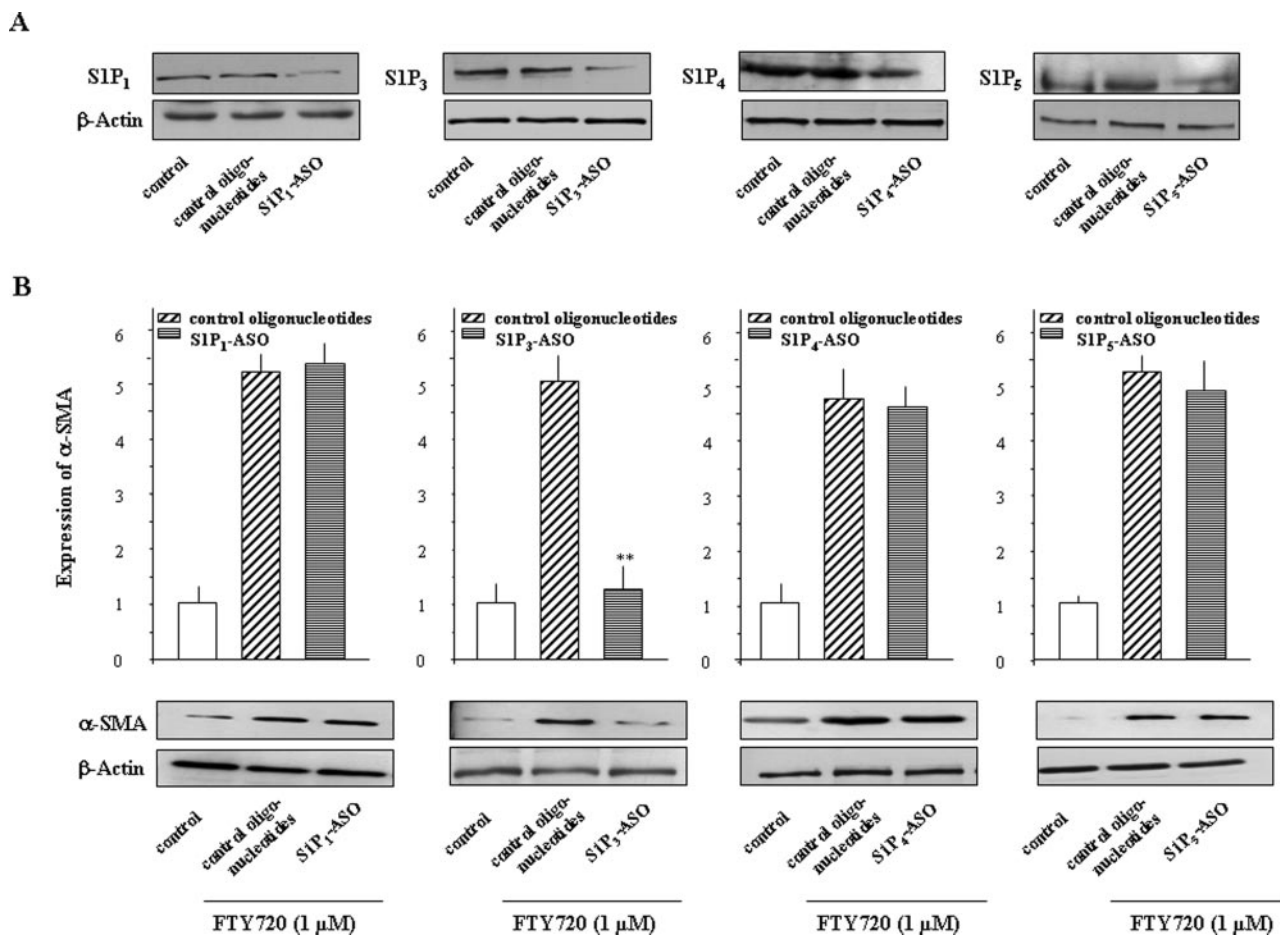


Figure 5. Involvement of S1P receptor subtypes in FTY720-mediated myofibroblast differentiation. Human fibroblasts were pretreated with control oligonucleotides (500 nmol/L) or S1P₁, S1P₃, S1P₄, or S1P₅-ASO (each 500 nmol/L) for 72 hours. **A:** Western blot analysis confirmed the relative abundance of the S1P receptor proteins expressed after ASO pretreatment. **B:** Then cells were treated with FTY720 (1 μ mol/L) for 72 hours, and α -SMA was measured by Western blot analysis and densitometric quantification normalized to β -actin. All results were confirmed in three independent experiments. ** $P < 0.001$.

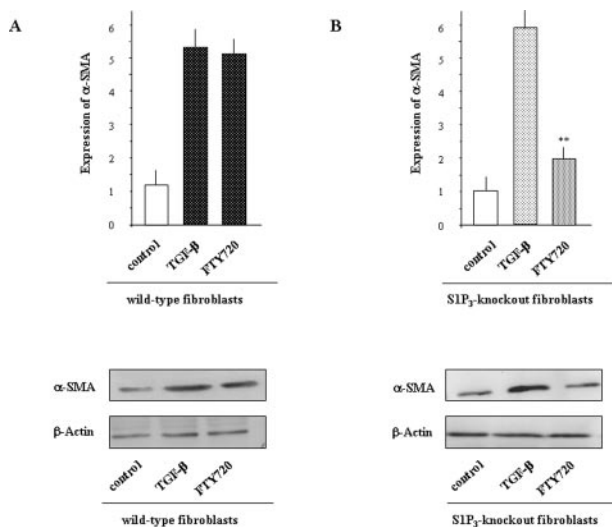


Figure 6. Myofibroblast differentiation of wild-type and S1P₃-deficient fibroblasts in response to FTY720 and TGF- β . Fibroblasts isolated from wild-type (**A**) and S1P₃^{-/-} mice (**B**) were stimulated with FTY720 (1 μ mol/L) or TGF- β (2 ng/ml) for 48 hours. Then α -SMA formation and its quantification normalized to β -actin were measured. All results were confirmed in three independent experiments. ** $P < 0.001$.

showing that diverse pharmacological actions of S1P are the result of a cross-communication between S1P receptors and other plasma membrane receptors.^{30–33} Such an interaction has also been indicated for TGF- β and S1P receptors in different cell types, which is accompanied by an activation of intracellular Smad proteins not only in response to TGF- β but also to S1P.^{11–13} Thus, S1P mimics the anti-inflammatory effect of TGF- β on mesangial cells because both mediators inhibit cytokine-induced secretion of phospholipase A₂, release of NO, and expression of matrix metalloproteinase 9 (MMP-9).¹³ Besides its role as an anti-inflammatory cytokine, TGF- β has also been identified as an important mediator in a number of fibrotic diseases, and increased levels of TGF- β are often present in tissues such as skin, lung, liver, kidney, and eye, where an uncontrolled fibrotic response takes place.^{34–36} The prominent role of TGF- β in fibrosis has been confirmed in several animal models of fibrotic diseases.³⁷ Thus, application of anti-TGF- β antibodies reduces kidney, lung, and skin fibrosis and administration of soluble TGF- β receptor II (T β RII) is beneficial in hepatic and intestinal fibrosis.^{37,38} Smad3 especially has been identified as an important mediator of pathological

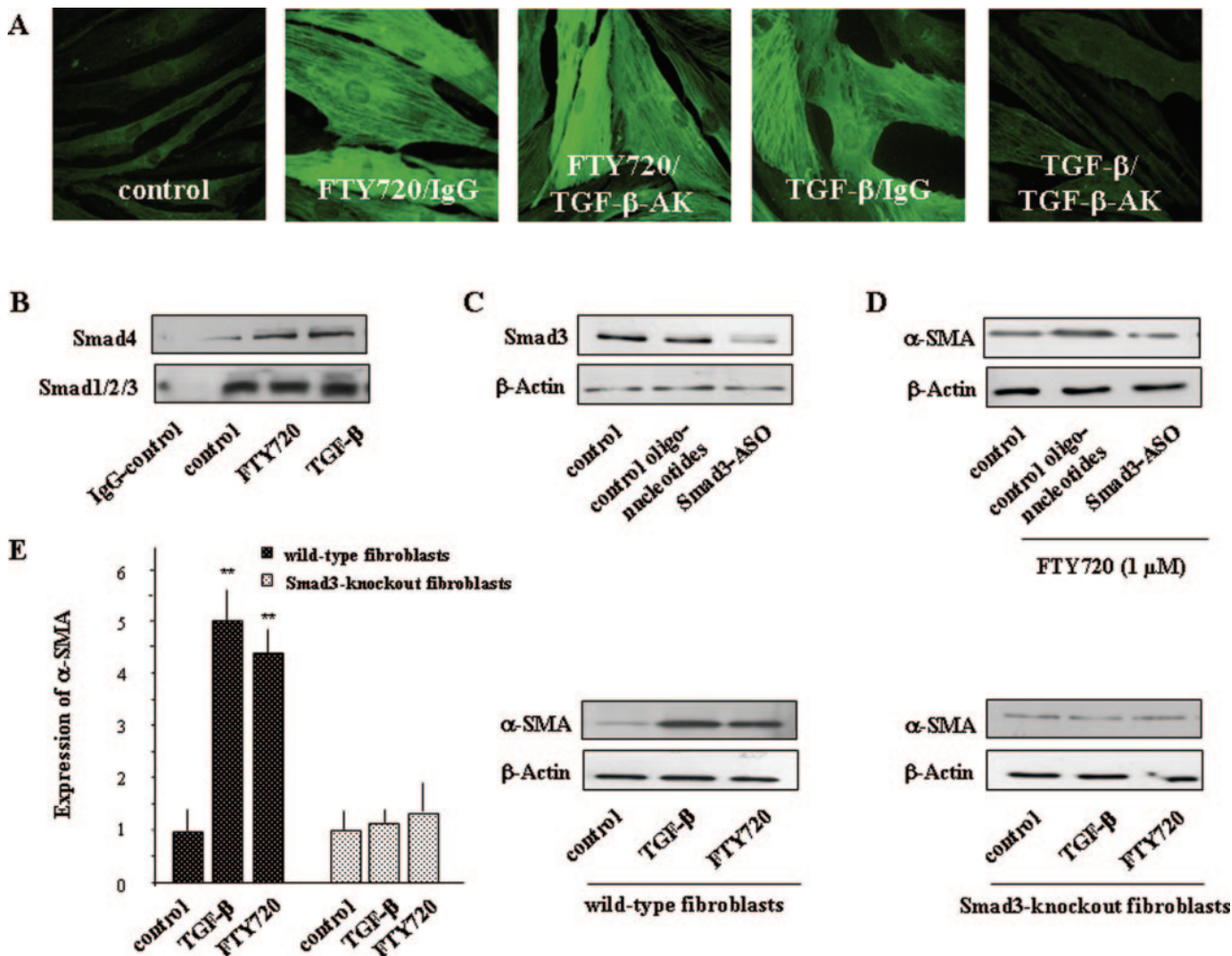


Figure 7. FTY720-mediated myofibroblast differentiation is dependent on activation of Smad3, but independent on TGF- β secretion. Cells were treated with normal goat IgG (25 μ g/ml) for control experiments or anti-TGF- β antibodies (25 μ g/ml). **A:** Then cells were stimulated with TGF- β (2 ng/ml) or FTY720 (1 μ mol/L) for 72 hours, and α -SMA was determined by immunofluorescence. Fibroblasts were stimulated with FTY720 (1 μ mol/L) or TGF- β (2 ng/ml) for 30 minutes. Lysates were immunoprecipitated with anti-Smad1,2,3 antibodies or with normal goat IgG (IgG control). **B:** Western blot analysis of immunoprecipitates was performed with anti-Smad1,2,3 (bottom) or anti-Smad4 antibodies (top). **C:** Cells were transfected with Smad3-ASO (500 nmol/L) or control oligonucleotides (500 nmol/L) for 72 hours followed by Western blot analysis of Smad3. **D:** Transfected cells were treated with FTY720 (1 μ mol/L) for 72 hours and α -SMA was determined by Western blot analysis. Fibroblasts isolated from wild-type and Smad3^{-/-} mice were stimulated with FTY720 (1 μ mol/L) or TGF- β (2 ng/ml) for 48 hours. **E:** Then α -SMA formation and its quantification normalized to β -actin were measured. All results were confirmed in three independent experiments. ** $P < 0.001$. Original magnifications, $\times 400$.

fibrotic conditions induced by TGF- β .^{15,36} In particular, TGF- β autoinduction is required for the recruitment of fibroblasts into the fibrotic area.^{15,35} Moreover, it has been suggested that Smad3 activation is responsible for the differentiation of fibroblasts into myofibroblasts, which then produce high amounts of extracellular matrix proteins.³⁹ In this context it should be noted that also S1P has been shown to induce differentiation of lung fibroblasts into myofibroblasts.⁴⁰ Therefore, it was of great interest to measure whether the sphingosine analogue FTY720 is also able to initiate transdifferentiation of fibroblasts.

Indeed, our results clearly indicate that FTY720 as well as the phosphorylated compound FTY720-P are potent inducers of myofibroblast differentiation. Although α -SMA expression as marker for the differentiation process was enhanced with the same potency by both compounds, it seems likely that conversion of FTY720 to

FTY720-P is necessary to mediate the differentiation process. Our data reveal that FTY720 loses its ability to increase α -SMA expression in the presence of the sphingosine kinase inhibitor DMS. Moreover, it has been suggested that FTY720 is mostly phosphorylated by the sphingosine kinase subtype SphK2.⁴¹ In SphK2 knockout mice, administration of FTY720 does not induce lymphopenia, whereas a direct dosage of FTY720-P induces a transient lymphopenia in this strain, indicating that SphK2 is constantly required to maintain FTY720-P levels *in vivo*.⁴² This is coherent with our data suggesting that FTY720 is not able to induce myofibroblast differentiation, when SphK2 expression is suppressed in human fibroblasts by the use of SphK2-ASO. Furthermore, radioligand competition assays of FTY720 with [³³P]S1P showed an IC₅₀ value of 300 nmol/L for the S1P₁ receptor, but no specific binding affinities of FTY720 were detectable at the other S1P receptor subtypes.^{5,6} Thus,

our data suggest that the receptor subtype S1P₃ is responsible for the FTY720- and FTY720-P-mediated transdifferentiation of fibroblasts because abrogation of S1P₁, S1P₄, and S1P₅ by specific ASO did not affect FTY720-induced α -SMA expression. In contrast, treatment of human fibroblasts with S1P₃-ASO did almost completely inhibit the FTY720-mediated transdifferentiation process. This result was also confirmed by the use of S1P₃ knockout fibroblasts. Furthermore, we provide evidence that Smad3 signaling is a crucial event for transdifferentiation of fibroblasts in response to stimulation of the S1P receptor subtype S1P₃. FTY720 not only induces activation of Smad signaling but also fails to induce transdifferentiation in Smad3 knockout fibroblasts. In this context it should be noted that Xin and colleagues⁴³ also present evidence that FTY720 and its phosphorylated analogue are able to phosphorylate Smad proteins in renal mesangial cells.⁴³ In this study, it has been shown that FTY720 up-regulates connective tissue growth factor and that this effect can be prevented by depletion of Smad4 using siRNA. Notably, connective tissue growth factor is a crucial mediator for the differentiation of fibroblasts into myofibroblasts.¹⁶

FTY720 is used in phase III clinical trials for the treatment of multiple sclerosis. Although it has been reported that bradycardia can occur in response to FTY720, less is known about other adverse effects like fibrosis.⁴ However, histological examination of liver and kidney from animals treated with repeat FTY720 for 1 or 3 weeks did not reveal any sclerosis, tubular changes, infiltrates, or fibrosis.^{44,45} Moreover, in a rat model of anti-Thy1-induced chronic progressive glomerulosclerosis, it has been demonstrated that FTY720 possesses an advantageous effect on the appearance of tubulointerstitial fibrosis.⁴⁶ However, it should be mentioned that in this rat model, infiltration of lymphocytes is a central event for the development of tubulointerstitial fibrosis. Because FTY720 induces sequestration of lymphocytes, a beneficial action can be expected in this model. Thus, it would be of interest to observe the action of FTY720 on fibrosis after long-term treatment.

Taken together, our results indicate that FTY720 induces transdifferentiation of fibroblasts into myofibroblasts. This effect is mediated by the phosphorylated compound of FTY720, which binds to the S1P₃ receptor resulting in the activation of Smad3 signaling. Because the lymphocyte sequestration is a consequence of S1P₁ activation, it should be considered whether a drug acting only on S1P₁ is more beneficial to avoid possible adverse effects.

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