2 MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Technical devices and consumptive materials

ABI PRISM 7900HT Sequence Detection System	Applied Biosystems, Darmstadt, D
ACT-1 software	Nikon, Düsseldorf, D
Agagel Standard	Biometra, Göttingen, D
Autoclave, Tuttaner-System Sterilizer 2540ELV	Guwina-Hofmann, Berlin, D
Cell Counter (0.0025mm2/0.1mm), Neubauer Improve1	VWR, Darmstadt, D
Centrifuge Eppendorf 5415D	Eppendorf, Hamburg, D
Centrifuge Megafuge 1.0R	Heraeus, Hamburg, D
Centrifuge Tubes (15 and 50 ml)	Sarstedt, Nümbrecht, D
Coverslips (round, Ø 18 mm)	Carl Roth GmbH, Karlsruhe, D
Crio Tube™ Vials	Nunc, Wiesbaden, D
Cuvettes (polystyrene, optical pathway 10 mm)	Sarstedt, Nümbrecht, D
Cuvette, UV (Plastibrand semi-micro)	Sarstedt, Nümbrecht, D
Developing Casket	Kodak, München, D
Digital still camera DMX1200	Nikon, Düsseldorf, D
Drying Chamber Function line B6	Heraeus, Hanau, D
Electrophoresis Chamber, Multigel long	Biometra, Göttingen, D
Film, Kodak X-OMAT, XAR-5	Sigma/Aldrich, Steinheim, D
Filtropur S 0.2 µm	Sarstedt, Nümbrecht, D
Flasks, TPP Tissue Cell Culture (25 and 75 cm^2)	TPP, Trasadingen, Ch
Incubator BB 6220	Heraeus, Hanau, D
Lamin Air–Sterile Working Bench HB2472	Heraeus, Hanau, D
Laser Scanning Microscope, LSM 510 Meta microscope	Carl Zeiss, Jena, D
Magnetic Agitator IKAMAG® RCT	Janke & Kunkel, Staufen, D
Needle (0.90 x 40 mm, 20 G x 1 ½”)	Carl Roth GmbH, Karlsruhe, D

Olympus BX41 microscope	Olympus, Hamburg, D
Omnifix® with needle (25G x 5/8”, 1 ml)	Carl Roth GmbH, Karlsruhe, D
Optical adhesive covers	Applied Biosystems, Darmstadt, D
Optical Reaction Plate (96-Wells)	Applied Biosystems, Darmstadt, D
PCR tubes (1.5 ml)	Eppendorf, Hamburg, D
Petri dishes, Falcon™ (10 cm)	BD Bioscience, Heidelberg, D
Phase Contrast Inverted Microscope, Axiovert 135	Carl-Zeiss, Jena, D
pH-Meter 766 Calimatec	Knick, Nürnberg, D
Pasteur pipettes	Carl Roth GmbH, Karlsruhe, D
Pipette Reference®	Eppendorf, Hamburg, D
Pipette Research®	Eppendorf, Hamburg, D
Pipettes, TPP serological (5. 10 and 25 ml)	Sarstedt, Nümbrecht, D
Pipetboy®	Integra Biosciences, Fernwald, D
Plates, Tissue culture (6-, 12- and 24-Wells)	TPP, Trasadingen, Ch
Polyvinyliden di-fluoride (PVDF)-Immobilon-P transfer membrane (0,45 µm pore size)	Millipore, Schwalbach, D
QuiaFilter Plasmid Maxi Kit	Quiagem, Hilden, D
QuickPrep™ mRNA Purification Kit	GE Healthcare Amersham Bioscience, Munich, D
Reagenzgläser, Pyrex ,16 x 100mm	Dunn Labortechnik, Asbach, D
Rocking platform WT16	Biometra, Göttingen, D
Safe-Seal microtubes (0.5, 1.5 and 2 ml)	Eppendorf, Hamburg, D
Scrapers, Tissue cell	TPP, Trasadingen, Ch
Shake Machine KS10, rotary	Gerhardt, Bonn, D
Shaker IKA® MT-2	Janke & Kunkel, Staufen, D
Slides, microscope	Menzel-Gläser, Braunschweig, D
Slides castes, microscope	VWR, Darmstadt, D
Standard Power Pack P25	Biometra, Göttingen, D
Syringe (5 and 20 ml)	Carl Roth GmbH, Karlsruhe, D
Tank–Blot	Biometra, Göttingen, D
Thermoblock TB 1	Biometra, Göttingen, D

Tips, pipette (10, 100, 1000 µl)	Sarstedt, Nümbrecht, D
Tips, Biosphere® (10, 100, 1000 µl with filter)	Sarstedt, Nümbrecht, D
Tissue Culture Inserts (Polycarbonate membrane, 8 µm pore size)	Nunc, Wiesbaden, D TPP, Trasadingen, Ch
Ultrashall bath Sonorex® RK 100	Bandelin, Berlin, D
UV-Photometer, Gene-Ray	Biometra, Göttingen, D
Vacuum Set Vacuboy®	Integra Biosciences, Fernwald, D
Vortex	Heidolph, Kellheim, D
Water Bath DC3/W26	Haake, Karlsruhe, D

2.1.2. Reagents

1,4-Dithiothreitol, DTT (30fold)	New England Biolabs, Frankfurt an Main, D
1,4-Dithiothreitol, DTT (0.1 M)	Invitrogen, Karlsruhe, D
2-Mercapto ethanol ≥98%	Sigma/Aldrich, Steinheim, D
Acrylamid/Bisacrylamid 40%, 29:1 (Rotiphorese® gel)	Carl Roth GmbH, Karlsruhe, D
Albumin, bovine fraction V powder (fatty acid free)	Sigma/Aldrich, Steinheim, D
Alexa fluor® 594 goat anti-mouse IgG (H+L)	Invitrogen GmbH, Karlsruhe, D
Amonium persulfate ≥98%	Sigma/Aldrich, Steinheim, D
Ampicillin, sodium salt	Sigma/Aldrich, Steinheim, D
Anti-goat antibody, bovine HRP-linked IgG (H+L)	Santa Cruz Biotechnology, Heidelberg, D
Anti-mouse antibody, horse-HRP-linked IgG (H+L)	New England Biolabs, Frankfurt am Main, D
Anti-rabbit antibody, goat-HRP-linked IgG (H+L)	New England Biolabs, Frankfurt am Main, D
Aprotinin	Sigma/Aldrich, Steinheim, D
Boric acid	Sigma/Aldrich, Steinheim, D
Calcium chloride ($CaCl_2$)	Fluka BioChemika, Buchs, Ch
Chloric acid (HCl)	Sigma/Aldrich, Steinheim, D
D-erythro Sphingosine 1-phosphate (S1P)	Biomol, Hamburg, D
Developer and replenisher (Kodak)	Sigma/Aldrich, Steinheim, D

Diethylpyrocarbonate, DEPC	Sigma/Aldrich, Steinheim, D
Dimethyl sulfoxide, DMSO	Sigma/Aldrich, Steinheim, D
dNTP Set (10mM pH 7,0)	Abgene, Hamburg, D
Dulbecco`s Modified Eagle`s Medium nutrition mixture F 12 Ham (15 mM HEPES)	Sigma/ Aldrich, Steinheim, D
Dulbecco´s Modified Eagle`s Medium, DMEM	Sigma/Aldrich, Steinheim, D
Epidermal Growth factor, EGF	Sigma/Aldrich, Steinheim, D
ERK1 (C-16) antibody, polyclonal rabbit IgG	Santa Cruz Biotechnology, Heidelberg, D
Ethanol, absolute extra pure	Merck, Darmstadt, D
Ethylendiamin tetraacetic acid (EDTA)	Sigma/Aldrich, Steinheim, D
Ethylenglycol-glycol-bis-(2-aminoethyl)-N,N,N',N'-tetra- acetic acid (EGTA)	Sigma/Aldrich, Steinheim, D
Fibronectin, human	Sigma/Aldrich, Steinheim, D
First Strand Buffer (5fold)	Invitrogen GmbH, Karlsruhe, D
Fixer and replenisher (Kodak)	Sigma/Aldrich, Steinheim, D
FuGene6™	Roche Diagnostic, Mannheim, D
Gentamicin sulfate	Biochrom, Berlin, D
Giemsa stain, modified	Sigma/Aldrich, Steinheim, D
Glycerol	Sigma/Aldrich, Steinheim, D
Glycerol phosphate	Sigma/Aldrich, Steinheim, D
Glycine	Sigma/Aldrich, Steinheim, D
HA-Fluorescein antibody, rat monoclonal IgG (Fab), clon 3F10	Roche Diagnostic, Mannheim, D
HA-probe (Y-11) antibody, rabbit polyclonal IgG	Santa Cruz Biotechnology, Heidelberg, D
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1M	Biochrom, Berlin, D
Igepal CA630	Sigma/Aldrich, Steinheim, D
Isopropanol (≥99%)	Sigma/Aldrich, Steinheim, D
Leupeptin	Sigma/Aldrich, Steinheim, D
L-Glutamin	Sigma/Aldrich, Steinheim, D
LumiGlo Chemiluminescence Reagent and peroxide	New England Biolabs, Frankfurt am Main, D
Luria Agar (Miller´s LB Agar)	Sigma/Aldrich, Steinheim, D
Luria Broth	Sigma/Aldrich, Steinheim, D

Lysophosphatidic acid	Sigma/Aldrich, Steinheim, D
Magnesium chloride (MgCl2)	Abgene, Hamburg, D
Manganesum chloride ($MnCl_2$)	Fluka BioChemika, Buchs, Ch
Methanol, LiChrosolv®, gradient grade	Merck, Darmstadt, D
Milk powder Sucofin (dry, low fat)	Trade Service International, Zeven D
Molecular biology grade water	Eppendorf, Hamburg, D
Mowiol	Merck, Darmstadt, D
N,N,N`,N`-Tetramethylenethylendiamin, TEMED	Sigma/Aldrich, Steinheim, D
Nitrogen, liquid	Praxair, Düsseldorf, D
Oligo(dT) $_{12-18}$ Primer	Invitrogen, Karlsruhe, D
Oligodesoxynucleotides, ODN	TIB Molbiol, Berlin, D
OptiMEM® Gibco®	Invitrogen, Karlsruhe, D
Oregon green® 488 phalloidin	Invitrogen, Karlsruhe, D
Ortho-phosphoric acid (H3PO4), 85%	Fluka BioChemika, Buchs, Ch
p44/42 MAP Kinase (ERK1/2), phospho (Thr202/Tyr204) antibody, mouse monoclonal IgG (E10)	Cell Signaling Technology by New England Biolabs, Frankfurt an Main, D
Paraformaldehyde	Merck, Darmstadt, D
PD098059	Sigma/Aldrich, Steinheim, D
Penicillin	Sigma/Aldrich, Steinheim, D
Pepstatin A	Sigma/Aldrich, Steinheim, D
Pertussis toxin, PTX	Merck, Darmstadt, D
Phenylmethylsulfonylfluoride (PMSF)	Sigma/Aldrich, Steinheim, D
Phosphate-buffered Saline Solution, calcium- and magnesium-free	Sigma/Aldrich, Steinheim, D Biochrom, Berlin, D
Piperazine-N, N'-bis [2-ethanesulfonic acid], PIPES, ≥ 99.5%	Sigma/Aldrich, Steinheim, D
Potassium chloride, KCl	Merck, Darmstadt, D
Potassium di-hydrogen phosphate, KH2PO4	Carl Roth GmbH, Karlsruhe, D
Potassium hydroxide (KOH)	Sigma/Aldrich, Steinheim, D
Primer sequences	TIB Molbiol, Berlin, DE
Protein marker broad range, prestained	New England Biolabs, Frankfurt an Main, D

Protein G-plus Agarose	Merck, Darmstadt, D
RNAsin ®, Ribonuclease-Inhibitor	MBI Fermentas, Leon-Rot, D
$S1P_1$ (P-20) antibody, goat polyclonal IgG	Santa Cruz Biotechnology, Heidelberg, D
$S1P_2$ (H-64) antibody, rabbit polyclonal IgG	Santa Cruz Biotechnology, Heidelberg, D
$S1P_3$ (H-15) antibody, goat polyclonal IgG	Santa Cruz Biotechnology, Heidelberg, D
$S1P_4$ (C-20) antibody, goat polyclonal IgG	Santa Cruz Biotechnology, Heidelberg, D
$S1P_5$ (G-13) antibody, goat polyclonal IgG	Santa Cruz Biotechnology, Heidelberg, D
SB431542	Sigma/Aldrich, Steinheim, D
SDS-Sample buffer, 3fold	New England Biolabs, Frankfurt an Main, D
Serum, fetal bovine	Biochrom, Berlin, D
Serva blue G	Serva Electrophoresis, Heidelberg, D
Smad1/2/3 (H-2) antibody, mouse monoclonal IgG_{2a}	Santa Cruz Biotechnology, Heidelberg, D
Smad2, phospho (Ser 465/467) antibody, rabbit polyclonal IgG	Invitrogen, Karlsruhe, D
Smad4 ((B-8) antibody, mouse monoclonal IgG_1	Santa Cruz Biotechnology, Heidelberg, D
SOB-Broth	Sigma/Aldrich, Steinheim, D
Sodium chloride (NaCl)	Sigma/Aldrich, Steinheim, D
Sodium deoxicholat monohydrat	Sigma/Aldrich, Steinheim, D
Sodium dodecyl sulfate (SDS)	Sigma/Aldrich, Steinheim, D
Sodium fluoride (NaF)	Sigma/Aldrich, Steinheim, D
di-Sodium hydrogen fosfate (Na2HPO4)	Merck, Darmstadt, D
Sodium hydroxide (NaOH)	Sigma/Aldrich, Steinheim, D
Sodium orto-vanadate (Na3VO4)	Sigma/Aldrich, Steinheim, D
β-Catenin antibody, mouse monoclonal IgG_1	BD Bioscience, Heidelberg, D
β -Actin (C4) antibody, mouse monoclonal IgG_1	Santa Cruz Biotechnology, Heidelberg, D
Streptomycin	Sigma/Aldrich, Steinheim, D
SuperScript™ II RNase H-Reverse Transcriptase	Invitrogen, Karlsruhe, D
SYBR Green Rox	Applied Biosystems, Darmstadt, D
Transforming Growth Factor-β 1, TGF-β 1	Sigma/Aldrich, Steinheim, D

TransIT®-LT1	KMF Laborchemie Handels, Lohmar, D
Tris Base (Trizma® Base) 99%	Sigma/Aldrich, Steinheim, D
Tris-HCl (Trizma® HCl) 99%	Sigma/Aldrich, Steinheim, D
Triton X-100	Sigma/Aldrich, Steinheim, D
Trypsin	Biochrom, Berlin
Tween® 20	Sigma/Aldrich, Steinheim, D
Vancomycin	Sigma/Aldrich, Steinheim, D
Vinculin antibody, mouse monoclonal IgG	Sigma/Aldrich, Steinheim, D

2.1.3. Culture media and solutions

2.1.3.1. Cell culture media

Fibroblasts growth medium	500 ml	DMEM F12 Ham
	10% (v/v)	Fetal bovine serum (FBS)
	2 mM	L-Glutamin
	100 U/ml	Penicillin
	50 µg/ml	Streptomicin

The growth medium for the transfection procedure was supplemented with 15% FBS.

Fibroblasts Basal medium	500 ml	DMEM F12 Ham
	100 U/ml	Penicillin
	50 µg/ml	Streptomycin

2.1.3.2. Solutions for cell culture

Transport medium	50 µg/ml	Gentamicin sulfate
	50 µg/ml	Vancomycin
	ad	DMEM
Freezing medium	10% (v/v)	DMSO
	10% (v/v)	FBS
	ad	DMEM F-12 Ham

Phosphate buffered saline	0.2 g/l	KCl
	8.0 g/l	NaCl
	0.2 g/l	KH_2PO_4
	1.44 g/l	Na_2HPO_4
	ad	dd Aqua
Trypsin-EDTA solution	1.67 mg/ml	Trypsin
	0.67 mg/ml	EDTA
	ad	PBS
Stop medium	10% (v/v)	FBS
	100 U/ml	Penicillin
	50 µg/ml	Streptomycin
	ad	DMEM F-12 Ham

2.1.3.3. Cell lysis solution

RIPA – buffer (Radioimmunoprecipitationassay buffer)

1% (v/v)	Igepal CA-630
0.5% (w/v)	Sodium deoxycholate
0.1% (w/v)	SDS
1mM	EDTA
ad	PBS without Ca^{2+}, Mg^{2+}

Immediately before cell lysis RIPA-buffer was supplemented with protease and phosphatase inhibitors:

50 mM	NaF
1 mM	PMSF
1 mM	Na_3VO_4
1 µg/ml	Aprotinin (solved in dd Aqua)
1 µg/ml	Leupeptin (solved in dd Aqua)
1 µg/ml	Pepstatin (solved in 50 µl acetic acid in ethanol)

For activation of the Na_3VO_4 stock solution pH was adjusted to 10. The solution was boiled until it turned colorless and after cooling to room temperature the pH was readjusted to 10. The procedure was repeated until pH stabilised at 10.

2.1.3.4. Bradford solution (5fold):

125 mg	Serva Blue G
125 ml	Ethanol (96%)
250 ml	H_3PO_4 (85%)
125 ml	dd Aqua

The dye was diluted 1:5 with dd Aqua immediately before use.

2.1.3.5. Solutions for electrophoresis and western blot analysis

Reduction buffer

Immunoprecipitates:	667 µl	3 fold SDS
	33 µl	DTT
	ad to 1ml	dd Aqua
Cell lysates (1:10):	1 µl	DTT
	9 µl	3 fold SDS

Collecting gel buffer (pH 6.8):	60 g/l	Tris-Base
	ad	dd Aqua

1xCollecting gel:

2.3 ml	dd Aqua
0.4 ml	SDS (1% in dd Aqua)
0.8 ml	collecting gel buffer
4 µl	TEMED
20 µl	APS (0.1 g/ml in dd Aqua)
0.5 ml	Acrylamid/Bisacrylamid 40%

Separating gel buffer (pH 8.8):	224.8 g/l	Tris-Base
	ad	dd Aqua

1xSeparating gel (10% SDS):	5.4 ml	dd Aqua
	1.2 ml	SDS (1% in dd Aqua)
	2.4 ml	Separating gel buffer
	10 µl	TEMED
	60 µl	APS
	3.0 ml	Acrylamid/Bisacrylamid 40%

1xSeparating gel (7.5% SDS):

5.5 ml	dd Aqua
0.9 ml	SDS (1% in dd Aqua)
2.5 ml	separating gel buffer
10 µl	TEMED
60 µl	APS
3.1 ml	Acrylamid/Bisacrylamid 40%

Running buffer (10fold):

144 g/l	Glycine
30.2 g/l	Tris-Base
10 g/l	SDS
ad	dd Aqua

Running buffer was diluted with dd Aqua (1:10) and pH was adjusted to 8.3.

Blotting buffer (10fold):

144 g/l	Glycine
30 g/l	Tris-Base
ad	dd Aqua

Blotting buffer was diluted with dd Aqua (1:10) and pH was adjusted to 8.

TBS-Buffer (10x):

12.14 g/l	Tris-HCl
87.66 g/l	NaCl
ad	dd Aqua

For TBST 0.1% v/v, Tween 20 was added and pH was adjusted to 8.

Blocking buffer:

5% (w/v)	low fat dry milk powder
ad	TBST

Stripping buffer (pH 6.8):

50 mM	Tris (HCl or Base)
100 mM	β-Mercaptoethanol
2% (w/v)	SDS
ad	dd Aqua

2.1.3.6. Antibodies solutions

Immunoprecipitation:

2 µg Smad 1/2/3 (200 µg/ml)/
ml Lysat (1-2 mg protein)
2 µg HA-probe (200 µg/ml)/
ml Lysat (1-2 mg protein)

Western blot:

Primary antibodies solutions:		
(1:1000)	0.2 µg/ml	anti-Smad1/2/3 (200 µg/ml)
	0.2 µg/ml	anti-Smad4 (200 µg/ml)
	0.25 µg/ml	anti-phosphoSmad2(250 µg/ml)
	0.2 µg/ml	anti-$S1P_{1-5}$ (200 µg/ml)
	0.25 µg/ml	anti-β-Catenin (250 µg/ml)
	0.2 µg/ml	anti-β-Actin (200 µg/ml)
	0.2 µg/ml	ERK1 (200 µg/ml)
	0.2 µg/ml	HA (200 µg/ml)
(1:2000)	0.5 µl/ml	anti-phospho ERK1/2
	each in 20 ml	dd Aqua

Secondary antibodies solutions (1:1000):

	1 µl/ml	anti-mouse-IgG
	0.4 µg/ml	anti-goat-IgG (400 µg/ml)
	1 µl/ml	anti-rabbit-IgG
	each in 20 ml	dd Aqua
Immunofluorescence:	4 µg/ml	anti-HAFluorescein (200 µg/ml)
	25 µl/ml	Oregon green phalloidin (300 units)
	2.5 ml/ml	anti-Vinculin
	5 µg/ml	Alexa fluor 594-IgG (2 mg/ml)
	each in 0.1 ml	PBS

2.1.3.7. Oligonucleotides and Primer

DEPC water	0.1% (v/v)	DEPC
	in	deionised Water over night.
	Sterilised by autoclave	
TE-Puffer (pH 7.6)	10 mM	Tris
	1 mM	EDTA
	ad	dd Aqua

Oligodesoxinucleotides (ODNs) sequences

Antisense techniques were carried on through coupling specific antisense phosphotioate oligonucleotides to the translational site of the target mRNA. Sequences are indicated in Table 1. ODNs were disolved in DEPC water and synthetised as follows:

Synthesis scale: 0.2 µmol
OD260: 32.5

Product	Antisense ODN	Scrambled ODN
$S1P_1$	5'-gacgctggtgggccccat-3'	5'-atggggcccaccagcgtc-3'
$S1P_2$	5'-gttgagcagggaattcagggtggaga-3'	5'-catcactagccacttgaagcaggcca-3'
$S1P_3$	5'-atggcaactgccctcccg-3'	5'-cgggagggcagttgccat-3'
$S1P_4$	5'-gaaggccagcaggatcatcagcac-3'	5'-acctagccaaccctccatgaaggc-3'
$S1P_5$	5'-gcaacaacataacgggccagcag-3'	5'-gcaacaacataacggg-3'

Table 1 Oligodesoxinucleotides sequences used for the antisense experiments.

Primer sequences

Specific primers against the five human S1PRs mRNA were create using the Primer3 programme (Table 2). Primers were synthesized as follows and dissolved in molecular grade water to a final concentration of 10 µM.

Synthesis scale: 0.01 µmol
Condition: 5 nmol
Lyophilized

Product	Left primer	Right primer	Size [bp]
$S1P_1$	5'- attactttaactggtagggaacg –3'	5'- aagacatctctcggtttaattgc –3'	152
$S1P_2$	5'- ggccttcgtagccaatacct –3'	5'- tgccatacagcttgaccttg -3'	173
$S1P_3$	5'- gccaccatttccactaggag -3'	5'- gcatattggtgcacattggt -3'	168
$S1P_4$	5'- gagagcaccctggtgtgg -3'	5'- catgatcgaacttcaatgttg c -3'	158
$S1P_5$	5'- ccacgactgtcttcccaagt -3'	5'- caagcagaacgtcaattcca -3'	179
GADPH	5'- atgcaacggatttggtcgtat -3'	5'- tctcgctcctggaagatggtg -3'	221

Table 2 Specific primer sequences selected from the human sphingolipid G protein-couple receptor mRNAs.

2.1.3.8. mRNA transcription solutions

Stock solution for cDNA synthesis

4 µl	5fold First strand buffer
2 µl	0.1 M DTT
1 µl	dNTP-Mix (dATP, dTTP, dGTP and dCTP, each 10 mM)
1 µl	RNAsin™ (Ribonuclease inhibitor)
1 µl	(200 units) SuperscriptTM II Reverse Transcriptase (added immediately before use)
	or
1 µl	molecular biology grade water for negative controls

TBE-buffer (5x, pH 8)	5.4 g/l	Tris-Base
	27.5 g/l	Boric acid
	2.92 g/l	EDTA
	in	dd Aqua

2.1.3.9. Plasmid amplification solutions

SOB-Medium	25.5 g	SOB Broth
	10 ml	250 mM KCl
	ad to 1 l	with dd Aqua
	pH was adjusted to 7 with 5 N NaOH	
	sterilised by autoclave	
TB-buffer	10 mM	PIPES
	15 mM	CaCl2
	250 mM	KCl
	pH was adjusted to 6.7 with KOH	
	55 mM	MnCl2
	sterilised by filtration	
LB-Medium	25 g	LB-Broth
	ad 1 l	dd Aqua
	sterilised by autoclave	
LB-Agar plates	40 g	Luria Agar
	ad 1 l	dd Aqua
	sterilised by autoclave	

TE-buffer	10 mM	Tris-HCl
	1 mM	EDTA
	ad	dd Aqua
	adjust to pH 8	

2.1.3.10. Solutions for Immunofluorescence

Fixing solution	4% (w/v)	para-formaldehyde
	in	PBS
Permeabilization solution	0.075% (v/v)	Triton X-100
	in	PBS
Blocking solution	1% (w/v)	BSA
	in	PBS
Embedding medium	6 g	Glycerol (analytical grade)
	2.4 g	Mowiol 4-88
	6 ml	dd Aqua

Add 12 ml 0.2 M Tris buffer pH 8.5 and mix for a half a day on a shaker. Let the mixture sit for 12 hours. Mowiol does not dissolve completely, therefore, incubate it at 50°C for 10 min (minutes) and centrifuge the mixture at 5000g for 15 min. Supernatants are aliquot and freeze at -20°C until use.

2.1.3.11. Potassium depletion solutions

Hypotonic medium	DMEM/H2O (1:1)	
Isotonic mediums:(A)	100 mM	NaCl
	in	50 mM HEPES, pH 7.4
(B)	100 mM	NaCl
	1 mM	CaCl2
	2.5% (w/v)	BSA
	in	50 mM HEPES, pH 7.4

10 mM KCl was added to the solutions to avoid intracellular potassium exchange

2.2. Methods

2.2.1. Isolation of fibroblasts

Foreskin fibroblasts cultures were established from newborn foreskins obtained from delivery suits of local hospitals. Tissues were delivered in transport medium at 4ºC and dissociated enzymatically by trypsin-EDTA for 30 min at 37ºC or stored at 4ºC in trypsin for the night. Stop medium was then added to finalize the enzymatic reaction. The cell suspension was spinned by 1000 rpm for 5 min at 4ºC. Isolated cells (passage 0) were collected and grown in DMEM with 10% FBS in 25 cm^2 flasks. Cells were pooled from at least three different donors.

2.2.2. Cell culture

Human fibroblasts, murine embryonic Smad3 KO and wild-type (WT) fibroblasts, and Swiss 3T3 fibroblasts were all maintained in DMEM F12 Ham containing 10% FBS and L-glutamine at 37ºC, 5% CO2 and 95% humidity. Confluent cells were splitted and seeded 1:4 in new 75 cm^2 flasks forming a new passage of cells. Only human primary cells of the 2nd or 3rd passage were used to all experiments. Optimal confluence of fibroblasts previous stimulation is 80%; if they grow more confluent, they form different layers one above the other, effect that is not appropiate to an efficient stimulation.

2.2.3. Test substances

S1P was solved in ethanol to make a stock solution with a concentration of $5x10^{-4}$ M and stored at -80°C. According to the requirements of the assay, it was further diluted with 0.4% BSA/PBS.

TGF-β was solved in 0.1% BSA in 4 mM HCl in PBS to a concentration of 1 mg/ml, aliquoted and stored at -80°C. This stock solution was diluted in 0.4% BSA in PBS directly before use.

EGF was solved in double distilled water (dd Aqua) to a final concentration of 10 µg/ml with subsequently store at -80°C. The stock solutions were further diluted in 0.4% BSA/PBS for each experiment.

Lyophilised PTX was solved in dd Aqua to a concentration of 100 µg/ml and stored at 4°C. For the assays, cells were incubated with 200 ng/ml PTX, dissolved in basal medium, for 2 h.

Solid anhydrous SB431542 was disolved at a concentration of 10 mg/ml in DMSO. Further dilutions of SB431542 in DMSO were made so that in all cases, SB431542 was added to cells at a final concentration of 10 µM for 30 min.

A 50 mM stock solution of PD098059 in dimethylsulfoxide (DMSO) was prepared, aliquoted and stored at -20°C. In order to prevent precipitating of the inhibitor the stock solution was diluted to 10 mM in DMSO immediately before test start. PD098059 was added to the cells at a final concentration of 50 µM for 1.5 h before the assay was performed.

The required final concentrations of all test substances were produced by dilution in the test medium. Control determinations contained solvents in the appropriate concentrations, but not exceeding 0.1% for DMSO (SB431542, PD098059) and 2% for 0.4% BSA in PBS (S1P, TGF-β).

2.2.4. Stimulation of cells

For detection of phosphorylated Smad2, ERK1/2 proteins, and detection of transfectants expression, fibroblasts ($2x10^5$/well) were seeded in 6-well plates and cultivated for 24 h. $1x10^6$ cells were seeded in 6 cm culture dishes to a proper isolation of cell membrane. For immunoprecipitation experiments $1.8x10^6$ fibroblasts were seeded in 10 cm culture dishes. Phosphorylation required incubation in serum-free medium the night before and 60 min treatment with 0.2 M HEPES-Buffer to suppress basal phosphorylation levels of negative control values. Cells were pre-treated with inhibitors followed by stimulation with control vehicle, 10% FBS, and the corresponding test substances, TGF-β or S1P.

2.2.5. Cell lysis and immunoprecipitation

After stimulation, solutions were aspirated and cells were rinsed twice with ice-cold PBS without calcium and magnesium. 100 µl/well (6-well plate) or 500 µl/well (10 cm culture dish) RIPA-buffer containing protease and phosphatase inhibitors were added and the plates shaken at 4°C until cell lysis could be observed under the light microscope. Then, cells were harvested by a cell scraper, transferred into 1.5 ml

centrifuge tubes, centrifuged at 14.000 rpm, 4°C for 30 min, and protein content was determined by Bradford staining (see chapter 2.2.7). The lysates could be stored at -80°C for several days. 20 µg of lysated protein were eluted in one-half volume of SDS-sample buffer supplemented with dithiothreitol (DTT 200 mM) and separated by SDS polyacrylamide gel electrophoresis. Detection of complex formation between R-Smad and Co-Smads or $S1P_1$ and TβR-I was worked out by immunoprecipitation experiments. After stimulation with TGF-β or S1P, cells were harvested in RIPA buffer. 1-2 mg of lysate protein were immunoprecipitated overnight at 4°C with 2 µg anti-Smad1/2/3 or anti-HA Probe antibodies. Fc-fragments of IgG were bound to protein G-plus agarose (4 µl/1µg antibody) by shaking at 4°C for 90 min. After centrifugation, the pellets were washed three times with 1 ml supplemented RIPA buffer and the immunoprecipitates were eluted and denaturated by boiling for 5 min in 60 µl SDS sample buffer with 200 mM DTT. Precipitated proteins were further processed by SDS-PAGE and western blot using an anti-Smad4 or anti-$S1P_1$ antibodies (20 ml, 1:1000 in dd Aqua) for detection. To determine levels of non-complexed Smad4 that were not precipitated with the anti-Smad1/2/3 antibody, supernatants after adsorption to protein G-Plus agarose were also immunoblotted with anti-Smad4 antibody.

2.2.6. Isolation of membrane proteins

Human fibroblasts grown and transfected in 75 cm^2 cell culture flasks were treated with pre-cooled membrane lysis buffer (20 mM Tris/HCl, 5 mM MgCl2, 1 mM EDTA, 0.6 mM EGTA freshly supplemented with protease inhibitors: 1 mM phenylmethylsulfonylfluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin and phosphatase inhibitors: 1 mM sodium orthovanadate, 50 mM sodium fluoride). Subsequent to sonification (3 times/10 sec), cell lysates were homogenised by use of a 0.9 mm followed by a 0.33 mm syringe. Cell scrap was removed by centrifugation at 2000 rpm/5 min/4°C. The supernatant was kept and the containing undissolved membrane proteins sedimented by centrifugation at 16000 rpm/60 min/4°C. The pellets were resuspended in ice-cold membrane lysis buffer and 20 µg aliquots were separated by SDS-gelelectrophoresis and detected by western blot analysis.

2.2.7. Determination of protein concentration by Bradford staining

Measurement of protein concentration in the cell lysates was performed by the Bradford dye assay (Bradford, 1976). It is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acid conditions, the dye is most stable as a doubly-protonated red form and upon binding to proteins it turns to an unprotonated, blue form.

A typical color response standard curve was established for bovine serum albumin (BSA) stock solution (50 µg/100 µl dd Aqua) (Table 3). The samples were prepared as follows:

BSA stock solution [µl]	BSA con-centration [µg]	RIPA-buffer [µl]	dd Aqua [µl]	Bradford solution [ml]
0	0	5	95	1
5	2.5	5	90	1
10	5	5	85	1
15	7.5	5	80	1
20	10	5	75	1

cell lysate [µl]	dd Aqua [µl]	Bradford [ml]
5	95	1

Table 3 Standard curve established for BSA with R^2 0.9. A linear regression of the data from the standard served to determine the protein cencentration.

Absorption was detected with a spectrometer at 595 nm. To determinate the protein concentrations of the original samples a linear regression of the data from BSA standard was conducted and evaluated according to Beer's law, which predicted that absorbance, is proportional to concentration:

$$A = Elc$$

A: Absorbance

E: Extinction coefficient for die substance being analysed

l: Path length of the cuvette

c: Concentration

According to Beer´s law, both variable (absorbance and concentration) are associated.

The regression equation follows the formula of a straight line:

$$y = m\,x + b$$

y: Absobance
m: Slope
x: Concentration
b: Intercept

Protein concentration was calculated as follows:

$$\Delta x = \Delta y / m$$

2.2.8. SDS-gel electrophoresis

Molecular weights:

Smad3	56 kDa	$S1P_1$	42.5 kDa
Smad2	58 kDa	$S1P_2$	41 kDa
Smad4	62 kDa	$S1P_3$	42 kDa
ERK1 (p44)	44 kDa	$S1P_4$	41 kDa
ERK2 (p42)	42 kDa	$S1P_5$	42 kDa
HA	55 kDa	β-Catenin	92 kDa
β-Actin	43 kDa		

Protein purification was carried out by gel electrophoresis and runned on a 10% SDS-polyacrylamid separating gel, exept for detection of the complex R-Smads with Smad4 that were allowed to run slowlyer in a 7.5% gel, and a 5% SDS-polyacrylamid collecting gel. Running buffer was diluted with dd Aqua and the electrophoresis chamber was filled in with it. Previously to gel loading, the probes were heaten for 5 min at 95ºC in a reduction buffer. Separation was implemented at 35 mA in the collecting gel and 55 mA in the separating gel.

2.2.9. Western blot analysis

Separated proteins were now transferred overnight from gels onto Polyvinyliden fluorid (PVDF) membranes in a tank-blot system. After SDS electrophoresis, the gel was carefully removed and inserted in blotting buffer for at least 15 min. The

membranes were wet with methanol (15 sec), washed with dd Aqua for 2 min, and kept in blotting buffer for at least 5 min. Gels and corresponding membranes were sandwiched in the blotting cassette outward sheltered by pre-wetted filter paper and cassette sponges. The stack was placed in the blotting container with blotting buffer and run over night at 100 mA. The next morning, blots were gently shaked for 1 h in 50 ml blocking buffer at 37°C on a rocker for saturation of unspecific binding sites. After three 5 min-washing steps with TBST the target proteins were marked by probing with the primary antibody and gently shaked for 2 h at room temperature (anti-Smad4, -Smad1/2/3 -phospho-ERK1/2, -S1P receptors, -HA-probe, -β-actin, -ERK1, -β-Catenin) or over night at 4°C (anti-phospho-Smad2). The blots were washed again three times with TBST followed by incubation with the horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h, washed, and developed with LumiGlo reagent and peroxide solution according to the manufacturer´s protocol.

2.2.10. Stripping

For detection of β-Catenin proteins from isolated membrane, the antibodies were removed from the corresponding blots by stripping in 50 ml stripping buffer pre-heated at 50°C in a water bath for 20 min. After triple 10 min washing with TBST the membranes were blocked and reprobed with anti-β-Catenin antibodies and incubated for 2 h at room temperature. Procedure was continued as described above with HRP-secondary antibodies.

2.2.11. Migration assay

The chemotactic behavior of cells can be assayed in a transwell migration assay, where cells are separated from a test substance by a membrane (8 µM pore size). Chemotactically active compounds induce the migration of cells through the membrane into the compartment containing the chemotactic agent. In this work, migration of cells was performed in response to a gradient of S1P or TGF-β. Polycarbonate membranes were coated with a solution of 3 µg/ml human fibronectin for 1 hour and dried. Following serum starvation, fibroblasts were trypsinized, counted, and resuspended in basal media. Cells ($8x10^5$ in 500 µl media) were added to the upper chamber. The lower chamber, separated by the membrane, contained the test substances, S1P, TGF-β or vehicle solution in a final volume of 500 µl. The

cells were allowed to migrate for 4.5 h at 37°C in a humidified chamber with 4.8% CO_2. After the incubation period, the filter was removed and no migrated cells on the upper side of the filter were wiped with a cotton swab, while the migrated one were fixed with ethanol, stained with Giemsa solution (1:10), and counted with a microscope. For each determination, 10 randomly chosen fields were counted and averaged. To measure chemokinesis the chemokine gradient was disrupted, thereby adding control vehicle, S1P or TGF-β to the upper and lower chamber.
A comparison of the migratory response of cells has been made possible by calculating the migration index (MI). It is calculated by measuring the lengths that cells migrate in the presence and in the absence of a chemotactic factor (*q.v.* 2.2.19).
Some cells were transfected with a foreign DNA (plasmid DNA or ODNs) or pre-treated with inhibitors or PTX before the migration assay was performed.

2.2.12. Transient transfection of fibroblasts

A short stretch of amino acids of the human hemagglutinin protein (HA) [YPYDVPDYA] was introduced into TβR-I unrelated recombinant protein by a technique known as “epitope tagging”. It is a widely used method that allows the surveillance of the fusion protein with tag-specific monoclonal antibodies (Kolodziej and Young, 1991). In case of $S1P_1$-GFP, a vector including the sequences corresponding to the $S1P_1$ and the green fusion protein (GFP) were transfected.
Fibroblasts were seeded to 70% confluence in grown medium without antibiotics. The next day transfection of 1 µg DNA (GFP-$S1P_1$, HA-TβR-I) or 500 nmol ODNs was carried out in transfection medium using FuGENE6® (1 µg DNA: 2 µl FuGENE6®) or TransIT® (1 µg DNA: 3 µl TransIT®) as transfections reagent according to the manufacturer’s protocol. After 8 hours, the cells have already taken up the foreign DNA or ODNs and the medium was changed to 15% serum-growing medium. The next day, medium was changed again to normal growing medium and the corresponding transfection time was waited. ODNs needed for an efficient transient transfection 3 days whereas 24 hours were enough for plasmid DNA.

2.2.13. Antisense technique

The genetic information encoded by genes is expressed in proteins with the help of the messenger RNA (mRNA). As a targeted method of disrupting this process,

protein expression was blocked with reversed or "antisense" genes. Antisense oligodesoxinucleotides (ASO) are short sequences of single stranded DNA, most likely less than 30 base pairs, which are engineered through chemical synthesis. The ASO sequence is specifically made to be complementary to an intracellular targeted mRNA molecule, preventing the synthesis of protein by the mRNA. ASOs are designed to surround the translational initiation site, a place empirically known to be most effective for inhibition of gene expression. As referee, same-length but randomly scrambled control ODNs sequences are also synthesized. The ODNs have a high negative density charge; therefore, the mechanism for the travel through the plasma membrane has to be forced. The use of a cationic liposome like FuGENE6® or TransIT® transfection reagents increases the potency of the antisense ODNs within the cell.

Fibroblasts were grown to 50-70% confluence and ODNs (500 nM) were transfected for 3 days at 37°C in growing media using FuGene6® or TransIT® transfection reagent according to the manufacturer's protocol.

2.2.14. Plasmid amplification

One of the easiest ways to get large amounts of DNA is to place the desired DNA into bacteria, grow the bacteria, then harvest the bacteria, and isolate the DNA. Bacteria can maintain DNA as plasmids: circles of DNA that usually contain a gene that allows the bacterium to grow in the presence of an antibiotic.

Transformation is a process involving the introduction of a foreign plasmid into bacteria. Bacteria are treated so they will take the plasmid up into them and are called competent cells.

2.2.14.1. Preparation of Competent Bacteria (*Escherichia coli*)

Since DNA is a very hydrophilic molecule, it will not normally pass through a bacterial cell membrane. In order to make bacteria take up the DNA, they must first be made "competent". This is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium. DNA can then be forced into the cells by washing in cold, low salts buffers or by mixing with a solution of PEG/DMSO. Inoue "ultra-competent" method provides a well-established method for this purpose (Inoue et al., 1990).

Regular *E. Colis* (MW8 strains) grown in LB medium are not highly competent; they must then be made competent. Inoculate 50 ml SOB media with a single colony bacterium from a fresh plate. Shake it well at 18°C (room temperature, RT) until optical density measured at 600 nm (Photometer Uvikon 922) reached 0.6 (OD_{600} ~ 0.6). An OD of 0.6 would be ideal, but from 0.2 to 1.0 would also work well. (Alternatively, try diluting an overnight culture to OD=0.1 and then growing at RT for approximately six hours). Place the centrifuge tube on ice for 10 min and then spin it for 20 min at 4°C and 4300 rpm. The pellets were resuspended in 16 ml ice-cold TB and placed on ice for 10 min. Following spinning, the new pellets were collected again in 4 ml ice-cold TB and supplemented with sterile DMSO to a final concentration of 7% (~ 0.28 ml). After 10 min on ice, the probes were dispensed into freezing tubes and transfer immediately to -80°C.

2.2.14.2. Bacterial transformation

Transformation involves mixing competent bacteria with plasmid DNA and then selecting bacteria containing the plasmid using agar plates that contain the corresponding antibiotic. For our chemically competent cells, the heat shock procedure is convenient.

Competent *E. coli* cells (DH5 or previously made MW8 strains), which have been kept in freezer storage, will be thawed on ice. 1 µg of plasmid DNA was added in the pre-cooled tubes, together with 50 µl of the *E.coli* suspension and gently mixed. The probes were incubated on ice for 30 min, briefly heat shocked for 2 min at 42°C, and immediately placed on ice for 2 min. 500 µl of pre-warmed (37°C) LB-medium was added and incubated at 37°C for 60 min. The bacteria suspension was plated out on a nutrient agar, which contains 1 µl/ml ampicillin that selects for transformants and incubated overnight at 37°C. For control of cell viability one agar plate without antibiotic was prepared. Using *E. coli* without the plasmid as a negative control (-) and *E. coli* with the corresponding plasmid (Table 4) as a treated or positive (+) sample, it will be able to directly observe the transformation of bacteria to ampicillin resistance. The (+) strain will survive on the antibiotic containing agar plate due to its antibiotic resistance.

Plasmids:

Plasmid	Vector	Antibiotica resistence	Concentration [µg/ml]
HA-TβR-I	pcMV5	Ampicillin	100
GFP-S1P$_1$	pcDNA3.1(-)	Ampicillin	100

Table 4 Plasmid specifications.

2.2.14.3. Plasmid purification

Purification of plasmid DNA was achieved by use of the Qiafilter Plasmid Purification Maxi Kit. All of the following mentioned chemicals and materials are provided in the kit. This procedure is based on a modified alkaline lysis, followed by binding of plasmid DNA to an anion exchange resin under appropriate low salt and pH conditions.

A starter culture of 3 ml antibiotic containing LB-medium supplemented with the corresponding antibiotic (1µl/ml penicillin) in a 50 ml centrifugal tube was inoculated with a single colony from a selective plate and incubated for 8 h at 37°C with intense shaking. 250 ml selective LB-medium were inoculated with 400 µl starter culture and grown for 12 to 16 h at 37°C with vigorous shaking until an optical density of 0.6 is reached. The cells were transferred to 50 ml centrifugal tubes and harvested by centrifugation at 6000 rpm and 4°C for 30 min. The pellets were thoroughly resuspended in 10 ml per tube resuspension buffer (P1) supplemented with RNase A (100 µg/ml). Then 10 ml lysis buffer (P2) was added, mixed by inverting the tube 4 to 6 times and incubated at room temperature for 5 min. Cell lysis reaction was finished by addition of 10 ml pre-cooled neutralisation buffer (P3) and mixed by inverting the tube 4 to 6 times. The lysates were poured into the barrel of the Qiafilter cartridge and incubated for 10 min at room temperature. The cell lysates were filtered by use of a plunger into a Qiagen-tip which has been equilibrated before by applying 10 ml QBT-buffer. After the clear lysates have been adsorbed to the resin, the Qiagen-tip was washed twice with QC-buffer. 15 ml QF-buffer were used to elute the DNA from the resin. DNA was precipitated with 10.5 ml isopropanol. After centrifugation (6000 rpm/1 h/4°C) the pellet was washed with 5 ml 70% ethanol and centrifuged (6000 rpm/1 h/4°C). The air-dried pellet was dissolved in 200-300 µl TE-buffer.

To determine the yield, UV-absorption was measured at 260 and 280 nm and calculated according to the following equation.

$$C\ DNA\ [\mu g/ml] = 50 \times d \times A_{260}$$

d: dilution factor
A_{260}: absorption at 260 nm
A_{280}: absorption at 280 nm

The applied procedure provided an A_{260}/A_{280} ratio of 1.7 to 2.0 indicating sufficient purity. The purity of the plasmid DNA was further checked by gel electrophoresis on a 0.75% agarose gel.

2.2.15. Immunofluorescence staining

Human dermal fibroblasts were grown on glass coverslips. When transfection was required, cells were transfected as described before and set on basal medium the night before stimulation. After stimulation, cells were fixed in 4% para-formaldehyde and then permeabilized in 0.075% Triton X-100 for 3 min by RT. Following blocking with 1% BSA/PBS, cells were stained with the corresponding antibodies. To visualize the actin cytoskeleton, cells were incubated for 20 min with Oregon green 488 phalloidin and with anti-vinculin antibody (1:400) to stain for FA, followed by secondary anti mouse antibody conjugated with Alexa fluor 594 (1:400). To determine HA-TβR-I expression and internalization, fibroblasts were stained with anti HA-Fluorescein antibody (1:50) for 60 min at 4°C. To visualize GFP-$S1P_1$ expression and internalization no staining was necessary. Coverslips were then embedded in Mowiol and attached to microscopes slides.

2.2.16. Visualization of fluorescent proteins

Actin cytoskeleton and FA staining were visualized with a fluorescence microscope. Images were taken on Digital still camera DXM 1200 using ACT-1 software.
Fixed fibroblasts transient transfected with GFP-tagged $S1P_1$ and HA-tagged TβR-I were analysed by confocal laser scanning microscopy using a Zeiss LSM 510 Meta

microscope. The quantification of the fluorescence signals was analysed using the software version 3.2 SP2 of the microscope.

2.2.17. Analysis of mRNA transcription and amplification

2.2.17.1. mRNA purification

Expression of $S1P_{1-5}$ receptors mRNA was analysed by real-time Polymerase Chain Reaction. For that purpose, mRNA was isolated from human dermal primary fibroblasts using a QuickPrep mRNA Purification Kit (up to $5x10^7$ cells) according to the manufacturer's instructions.

The kit was firstly carried to room temperature. Cells from one 75 cm^2-culture flasks were treated with trypsin as already described, washed with PBS, and transferred to 1.5 ml PCR tubes and centrifuged. 1.5 ml guanidinium thiocyanate buffer (extraction buffer) containing RNAase inhibitors, was added to the pelleted cells. To assure complete homogenisation and disruption, the extract was passed through a 21-gauge needle attached to a syringe. To dilute the sample, 3 ml elution buffer were added to the extract and mixed thoroughly and the rest of elution buffer was warmed at 65°C.

The 1.5 ml centrifuge tubes were centrifuged at 14.000 rpm for 10 min. Meanwhile, the oligo(dT)-cellulose spun column was inverted several times previous use, centrifuged at 2000 rpm for 2 min and the liquid discarded. 4 ml of the sample were transferred to onto the surface of the resin of the oligo(dT)-cellulose spun column. The column was gently mixed by inverting the column manually for 10 min followed by centrifugation at 2000 rpm for 2 min to separate the resin from the suspension. The supernatant was decanted and discarded. The poly(A)+ RNA adsorbed to oligo(dT)-cellulose was washed three times with 3 ml high-salt buffer and two times with 3 ml low-salt buffer always followed by centrifugation at 2000 rpm for 2 min. A 1.5 ml microcentrifuge tube was placed inside a 50 ml centrifuge tube and the column was then inserted in such a way that the tip of the column was inside the microcentrifuge tube. Bound Poly(A)+ RNA was eluted using three washes each of with 0.25 ml pre-warmed elution buffer followed by centrifugation (2000 rpm 2 min).

The concentration of isolated mRNA in the final eluate was quantified by spectrophotometry. A quartz cuvette was incubated with concentrated HCl and methanol (1:1) to protect the RNA sample from degradation and washed with grade water before measurement. 0.4 ml mRNA solution was poured in a

spectrophotometer and absorbance was readed at 260 and 280 nm previously blanked against elution buffer.
The following formula calculates RNA concentration based on UV-absorption.

$$CRNA\ [\mu g/ml] = 40 \times Abs260$$
$$MassRNA\ [\mu g] = CRNA \times V$$

40 µg/ml = concentration for RNA with an absorbance of 1 at 260 nm
Abs_{260}: absorbance at 260 nm (wavelength of RNA)
$A_{260} / A_{280} \geq 1.8$
V = elution buffer volume

To guarantee high RNA purity the calculated Abs_{260}/Abs_{280} ratio is supposed to be ≥ 1.8.
To precipitate the isolated mRNA, 50 µl potassium acetate solution, 10 µl glycogen solution, and 1 ml ethanol (chilled to -20°C) was added to the sample and incubated at -20°C for a minimum of 30 min. After centrifugation (14.000 rpm/4°C/5 min) mRNA sample were further either processed or stored at -80°C.

2.2.17.2. Synthesis of complementary DNA (cDNA)

The mRNA probes either were defrosted or immediately centrifuged (14.000 rpm/4°C/30 min). Afterwards the mRNA pellets were dissolved in molecular biology grade water to a final concentration of 100 ng/µl, assuming that mRNA yields after precipitation were about the 50% of the initial purification.

$$V = Mass \times 20$$
$$V' = V/2$$

20 = mRNA/Reverse Transcriptase ideal ratio
V = volume of grade water for 100% mRNA purification
V' = volume of grade water assuming 50% of purification

1 µg mRNA was copied to cDNA by reverse transcriptase Superscript™ using an oligo(dT)14-18 primer. The reverse transcriptase enzyme is a RNA-dependent

polymerase that uses an mRNA template to form cDNA. This enzyme also digests and removes mRNA allowing the second strand of DNA to be formed. As other polymerases for synthesis of single stranded DNA do, a short double-stranded sequence is needed at the 3’ end of the mRNA, which acts as a start point for the polymerase reaction. This is provided by a short synthetic oligonucleotide (oligo (dT) primer) complementary to the poly(A) tail found at the 3' end of most eukaryotic mRNAs. Therefore, 200-500 ng mRNA solution and 1 µl (1 pmol) oligo(dt) primer were incubated at 80°C for 3 min and at 37°C for further 10 min. 9 µl of freshly prepared stock solution for cDNA synthesis was added to 10 µl of the RNA solution and reverse transcription took place by incubation for 90 min at 37°C. After addition of 20 µl 0.4 M NaOH the probes were incubated for 10 min at 37°C and afterwards, 20 µl 1M Tris-HCl was added and the solution was frozen at –20°C. DNA probes could be kept this way for several months.

2.2.17.3. Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

The polymerase chain reaction served to amplify the transcript cDNA previously obtained. A very sensitive method that provides a quantification of the expression of a protein is the real-time PCR. QRT-PCR assays were conducted using SYBR Green Master Mix on an ABI PRISM 7900HT Sequence Detection System according to the manufacturer’s protocol. Relative mRNA expression was quantified using the comparative threshold method according to the ABI manual. Data normalization was performed using Glyceraldehyde-3-phosphate dehydrogenase (GADPH) as a reference gene. Amplification was performed in a 96 well optical reaction plate in 10 µl-reactions containing 1fold SYBR Green Master Mix, 250 nM primer concentration, 5 ng of total cDNA, and dd Aqua ad to 10 µl. The thermocycling program consisted of 2 min at 50 C, 10 min at 95 C followed by denaturation at 95°C for 10 s and amplification by 40 cycles of PCR (95°C for 15 s, 60°C for 1 min). Dissociation curves were generated by first heating to 95°C (15 s), then cooling to 60°C with a 15-second hold, and finally heating at 0.1°C/s to 95°C with continuous fluorescence acquisition. Optical data were collected at the end of each extension step. In each case, product identity was demonstrated by the presence of a single peak on derivative melting curve plots. For each of the three replicates of a sample, the average cycle time (Ct) and the standard deviation were calculated. The average

Ct is normalized to the average Ct of the reference gene (GADPH) (ΔCt). The fold-induction for each sample relative to the reference gene resulted from $2^{(-\Delta Ct)}$. The resulting induction values were plotted as a bar graphic.

2.2.18. Potassium Depletion

Larkin et al. established a potassium depletion protocol to study several aspects of coated pit function. Potassium-depleted fibroblasts showed a significant inhibition of receptor-mediated endocytosis whereas coated pits were rapidly formed when these cells were subsequently incubated in the presence of 10 mM KCl (Larkin et al., 1986). According to his method, fibroblasts were treated as followed:
Cells were initially incubated for 5 min at 37°C in DME/H2O (1:1). After 5 min hypotonic shock, followed a 10-min incubation at 37°C in the isotonic medium A, and a 30-min in medium B. Control cells were treated as above, but did not undergo initial hypotonic shock. Subsequent incubations in buffers were performed in the presence of 10 mM KCl. Cells were depleted for 30 min prior to TGF-β or S1P treatment and then prepared for immunoprecipitation and western blotting. In experiments in which TβR internalization was monitored, cells were then fixed and stained as indicated for immunofluorescence.

2.2.19. Data analysis and statistics

This work was performed in human primary cells from different donors. Fibroblasts were originally isolated from human skin and then pooled from at least three different donors to obtain a genetically heterogenic culture of cells.

Since the cells used in every migration assay differ from each other, data normalization was performed using the migratory value in absence of stimuli as reference. Relative values of migration are represented as MI. This allows comparing the migratory response. The counted migration rates ($Average_{test}$) were referred to those of non-stimulated controls ($Average_{control}$), which were defined to have a migration index of one. MI were calculated as follows:

$$Migration\ Indices_{test} = \frac{Average_{test}\ x\ 1}{Average_{control}}$$

In case of regression analysis, the simple linear regression method was used defining the experimental variable (absorbance) and testing the response of another variable (concentration) to it. To test the statistical significance of the association of these two variables, the coefficient of determination (R^2) was examined. The coefficient of determination, gives the proportion of the variance (fluctuation) of one variable that is predictable from the other variable. R^2 was always about 0.9, which means that 90% of the total variation in the absorbance could be explained by the variation in the concentration.

The expression of the S1P receptors was performed in triplicate. Each time with three different mRNA, that was isolated from polled human fibroblasts cultures.

Data are presented as the arithmetic mean values ± standard deviation (mean ± SD). Data was tested for normal distribution by Shapiro-Wilk normality test. Since the cells were randomly assigned into different groups, the samples were considered as independent or not paired. For comparison of mean values, Student's t-test was performed if the F test assumed that the variances were equal. When this assumption was not fulfilled, in case of inhomogeneous variances, the Welch test was applied. A p value beneath the threshold chosen for statistical significance (*, $p \leq 0.05$ or **, $p \leq 0.005$) was considered to be significant.