3 RESULTS

3. RESULTS

3.1. Effects of S1P on the motility of human primary fibroblasts

3.1.1. Influence of S1P and TGF-β in the migratory activity of human fibroblasts

Chemotaxis and especially chemotactically active factors are not only important mediators of a number of physiological, but also pathological processes, including development, embryogenesis, immune responses, inflammation, wound healing, atherosclerosis, angiogenesis, and metastasis. Since the lysophospholipid S1P, as well as the growth factor TGF-β, have been identified as contributors to the regulation of migratory processes in some cell types, their migratory potency on primary dermal fibroblasts of human origin was examined. To this end, different concentrations of TGF-β and S1P were used as chemokines and the corresponding responses were measured.

It has already been reported that TGF-β induced reciprocal responses regarding some cellular effects according to cell type and concentration (Bandyopadhyay et al., 2006). In order to clarify the specific response of the growth factor, the motility of human fibroblasts towards TGF-β was first investigated. As the expression of TβRs is high in dermal fibroblasts (Bandyopadhyay et al., 2006), a signal activation upon agonist stimulation was expected. As seen in Fig. 10, TGF-β increased the migration of fibroblasts. Interestingly, high and low concentrations of TGF-β showed a less migratory activity than the middle one. Within the concentrations tested, the MI of human dermal fibroblasts showed its maximal response at 0.5 ng/ml TGF-β stimulation. As expected, cells potently migrated toward FBS, which has been applied as positive control for motility.

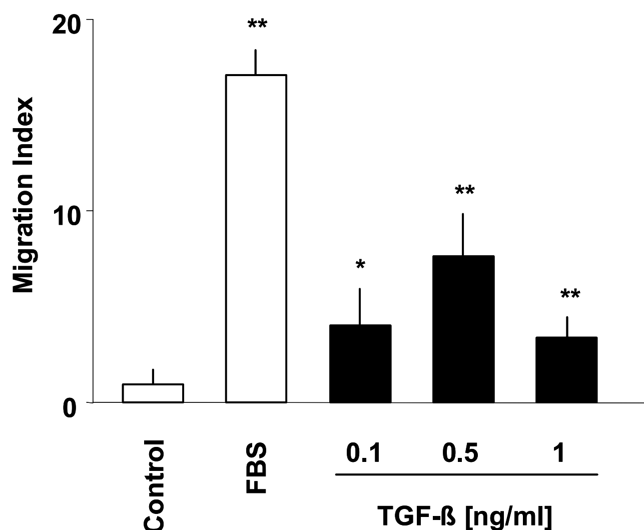


*Fig. 10 Influence of TGF-β on human dermal primary fibroblasts motility - Cells were allowed to migrate for 4.5 h in the presence of control vehicle (0.4% BSA/PBS), 10% FBS as positive control, and TGF-β at indicated concentrations. The data are expressed as means ± SD of duplicate determinations. Relative values of migration normalized using the migratory values in absence of stimuli are represented as MI. Similar results were obtained in two independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$.*

Then, the influence of the lysophospholipid S1P on the motility of human dermal fibroblasts was explored and values were related to the control values. For this purpose, different concentrations of S1P were added beneath the filter and migrated cells were counted after 4.5 hours. The migration assay resulted in a similar response compared to TGF-β. As shown in Fig. 11, the dose-dependent response of fibroblasts to S1P followed a bell-shaped curve with micromolar (10^{-5} M) and picomolar (10^{-10} and 10^{-12}) concentrations resulting in a loss of migration, a characteristic of most chemotactic factors (Yokomizo et al., 1997) (Kon et al., 1999). S1P revealed its maximal effect in a concentration range between 10^{-7} and 10^{-9} M. The peak of the migratory response of S1P was comparable to that of the positive control (10% FBS).

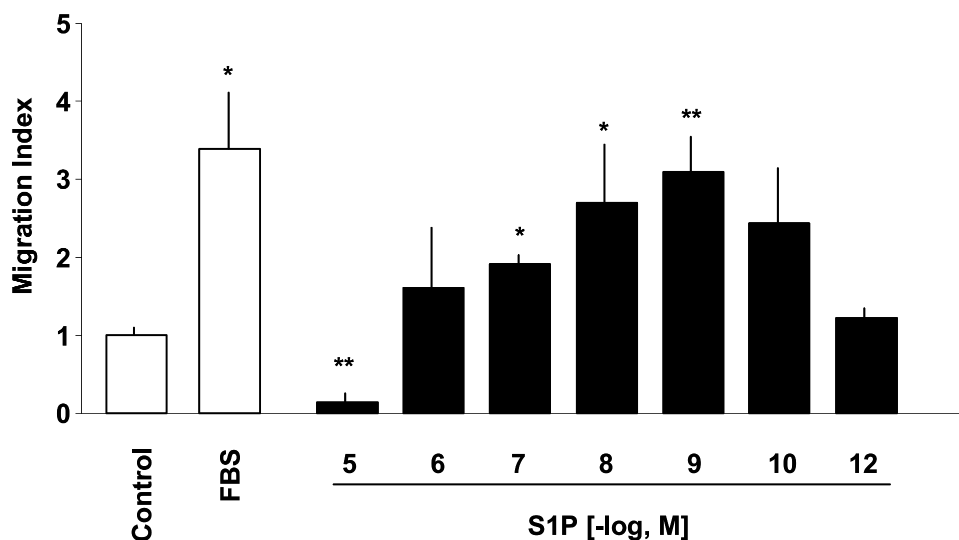


Fig. 11 *Effect of S1P on fibroblast motility - Primary cultures of human fibroblasts were allowed to migrate in a transwell assay toward control vehicle (0.4% BSA/PBS), FBS (10%) or different concentrations of S1P for 4.5 h. The data are expressed as means ± SD of triplicate determinations. Relative values of migration normalized using the migratory value in absence of stimuli are represented as MI. Similar results were obtained in three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$.*

3.1.2. Chemokinetic effect of the migratory response to a gradient of S1P

Because of the detected migratory response, it was necessary to differentiate the directed movement upon a chemoattractant, chemotaxis, from the non-directed movement or chemokinesis. To this purpose, two different preparations were necessary for each concentration of the stimulus. As described in chapter 2.2.11, in case of chemotaxis the stimulus was located beneath the filter, but when chemokinesis was measured, the created gradient of concentration had to be disrupted. If the fraction of random movement of the S1P-evoked migration had to be studied, the stimulus was located above the filter, together with the cell suspension, as well as beneath the filter. As seen in Fig. 12, a chemokinetic effect appeared at low concentrations of S1P (10^{-10} - 10^{-12} M). At these concentrations, the chemotactic bell-shaped curve was shifted due to a loss of the directed migration, which confirms the results seen in Fig. 11, but the random movement of the cells became increased. Hence, the remaining effect after the loss of chemotaxis was due to a randomized movement created when the chemokine gradient was missing.

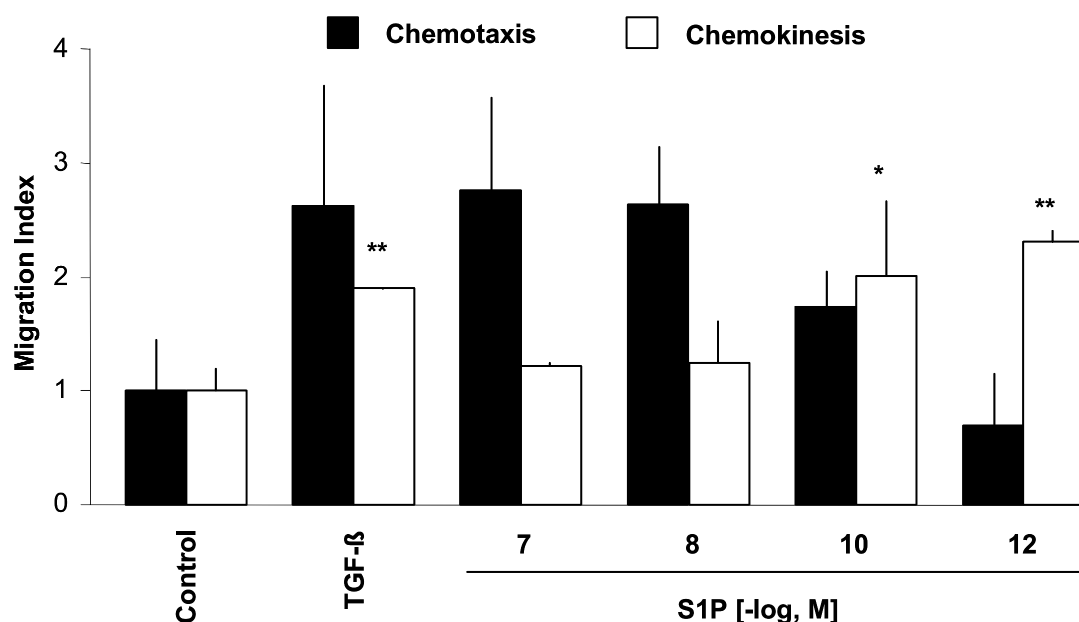


*Fig. 12 Chemokinetic effect of TGF-β and S1P-induced motility – Fibroblasts were allowed to migrate in a transwell assay in response to vehicle (0.4% BSA/PBS), TGF-β 1 ng/ml or different concentrations of S1P for 4.5 h. Chemokinesis assay was performed as described, thereby adding test substances to the upper and lower chamber (white bars). The data are expressed as means ± SD of duplicate determinations. Relative values of migration normalized using the migratory value in absence of stimuli are represented as migratory index. Similar results were obtained in three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.005$.*

This result indicated that the MI measured in Fig. 11 at low S1P doses was a result of chemokinesis and no longer to the chemotactic properties of the lysophospholipid. Interestingly, it was observed that 1 ng/ml TGF-β did not induce a significant chemotactic movement of human dermal fibroblasts, but rather a random one. The results differed between the chemotactic S1P concentrations 10^{-6} to 10^{-9} M and the motile concentrations 10^{-10} to 10^{-12} M that acted principally chemotactically (Fig. 12). Since the MI in the absence of stimulus is lower than when it is present, S1P was characterized as a chemotactic factor in a concentration range from 10^{-7} to 10^{-9} M.

3.1.3. Cytoskeletal changes caused by S1P to induce motility

Cell movement is basically directed by the complex interplay of actin formation at the leading edge of the cell and the continuous formation and disassembly FA. To

examine the capacity of S1P to induce those necessary events for motility, fibroblasts were double labelled for actin and vinculin respectively.

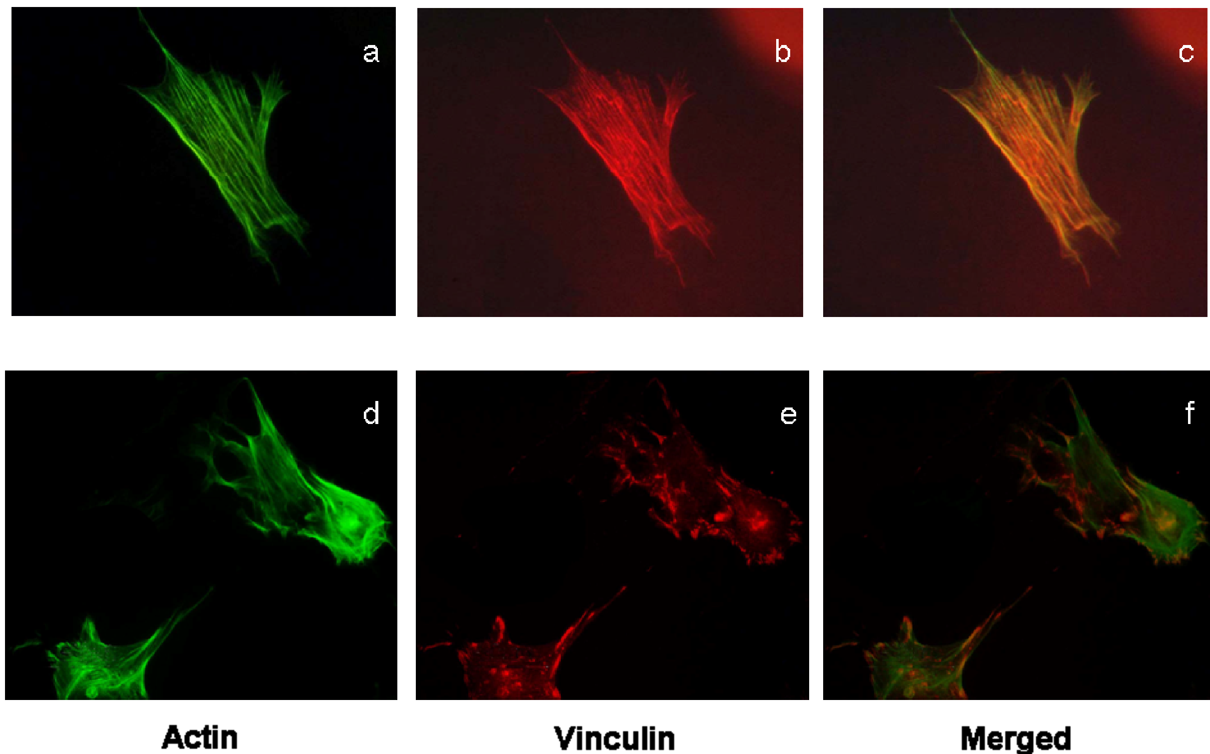


Fig. 13 *Changes in cytoskeletal architecture and focal complexes of fibroblasts induced by S1P - Primary cultures of human fibroblasts were grown on coverslips and then stimulated without (a,b and c) or with 10^{-7} M S1P (d, e and f) for 30 min. Cells were then fixed, permeabilized, and stained with Oregon green 488 phalloidin (a and d) to detect actin fibers (green) and anti-vinculin and Alexa fluor 594 antibodies (b and e) to detect adhesion complexes (FA) (red). The staining was visualized by fluorescence microscopy. Red and green colours in the merged pictures are derived from the images in the left and middle panels and showed the localization of both proteins in the same cell under basal conditions (c) or after stimulation (f).*

Since reorganization of the actin cytoskeleton is a substantial event in the initiation of cell migration, its architecture after S1P stimulation was firstly examined, and then analyzed together with FA formation. In basal unstimulated fibroblasts, cells showed a typical morphology for non-motile cells, which was characterized by stress fiber localization and homogeneity in vinculin staining (Fig. 13.a - c). On the contrary, S1P stimulation caused extension of lamellipodia at the cell periphery of fibroblasts and actin accumulation at the cell front concomitantly with a rapid turnover of FAs, marked by an irregular vinculin distribution (Fig. 13.d - f). The double staining for actin and vinculin revealed an association between the increased actin accumulation

and the increased FAs turnover upon S1P treatment. These results correlated with the significantly increased migration observed in Fig. 11.

3.1.4. Analysis of S1PRs expression in human fibroblasts

Each S1PR subtype signals through a unique set of G proteins that results in various downstream cellular effects. It has yet not been reported which S1PRs are present in human primary fibroblasts. In order to predict the cellular behavior upon S1P stimulation, it was critical to know the S1PR expression profile and to quantify the differences in mRNA expression. For that purpose, a real-time PCR was performed. Firstly, mRNA was isolated from three different pools of cells using the QuickPrep mRNA Purification Kit according to the manufacturer's instructions. Then, 1 µg isolated mRNA was copied to cDNA by reverse transcriptase Superscript™. Finally, quantification of S1PRs mRNA using SYBR® Green fluorescence and real-time PCR were carried out in the presence of specific small oligonucleotide DNA primers (Table 2).

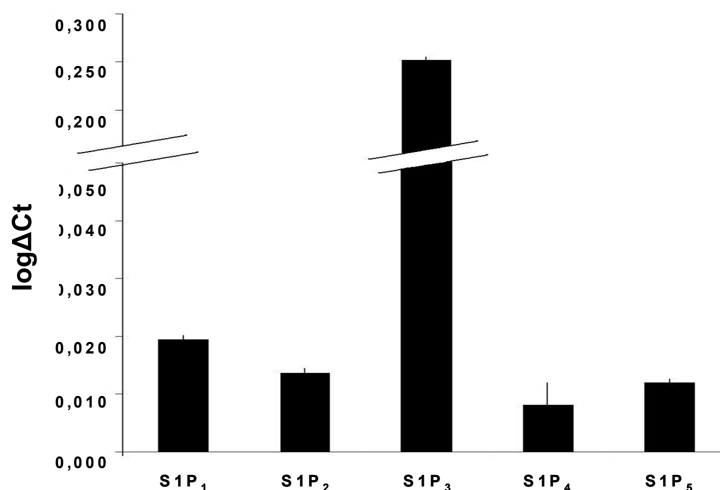


Fig. 14 Expression profile of S1PRs in human dermal fibroblasts by real-time PCR - The relative levels of the mRNA transcript are shown (S.D. from triplicates reading). Data normalization was performed using GADPH as reference gene. Similar results were obtained in three different experiments.

As seen in Fig. 14, analysis of mRNA transcripts of human fibroblasts indicated the expression of all known five S1P receptors ($S1P_{1-5}$). $S1P_3$ was predominantly expressed compared to $S1P_{1,\ 2}$, and $S1P_{4,\ 5}$, which showed comparable expression profiles.

3.1.5. Designation of a S1P receptor-mediated extracellular signal

It has been suggested that S1P mediates its action either extracellularly via S1PRs or intracellularly after being formed by SphK. Nevertheless, motility has been attributed to an extracellular signal of S1P in different cell types (Young and Van Brocklyn, 2006). Since dermal primary cells of human origin expressed all five S1P receptors ($S1P_{1-5}$), although not in the same proportion, the requirement of receptors for the migratory response induced by the lysophospholipid was firstly addressed. PTX specifically inactivates Gi-type heterotrimeric protein through ADP ribosylation. Therefore, the effects of pre-treatment with PTX on S1P-induced chemotaxis were assessed. Indeed, the stimulatory response to exogenously added S1P was completely sensitive to PTX (Fig. 15), indicating an involvement of Gi proteins coupled to S1P receptors in the migration process and excluding the idea of an intracellular mechanism of action.

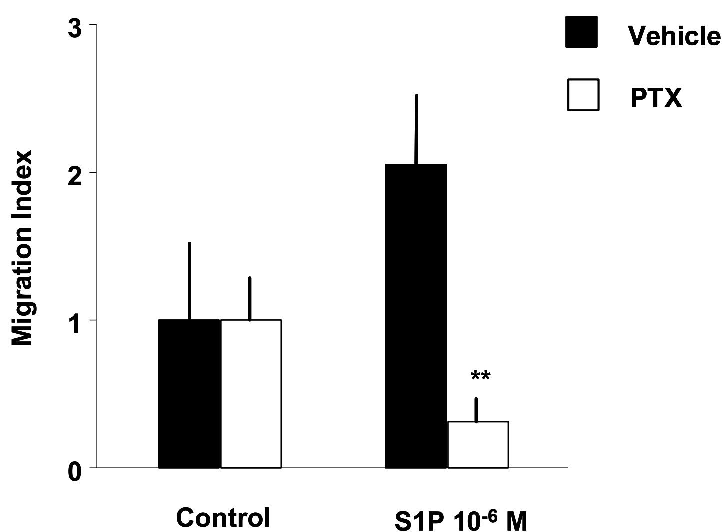
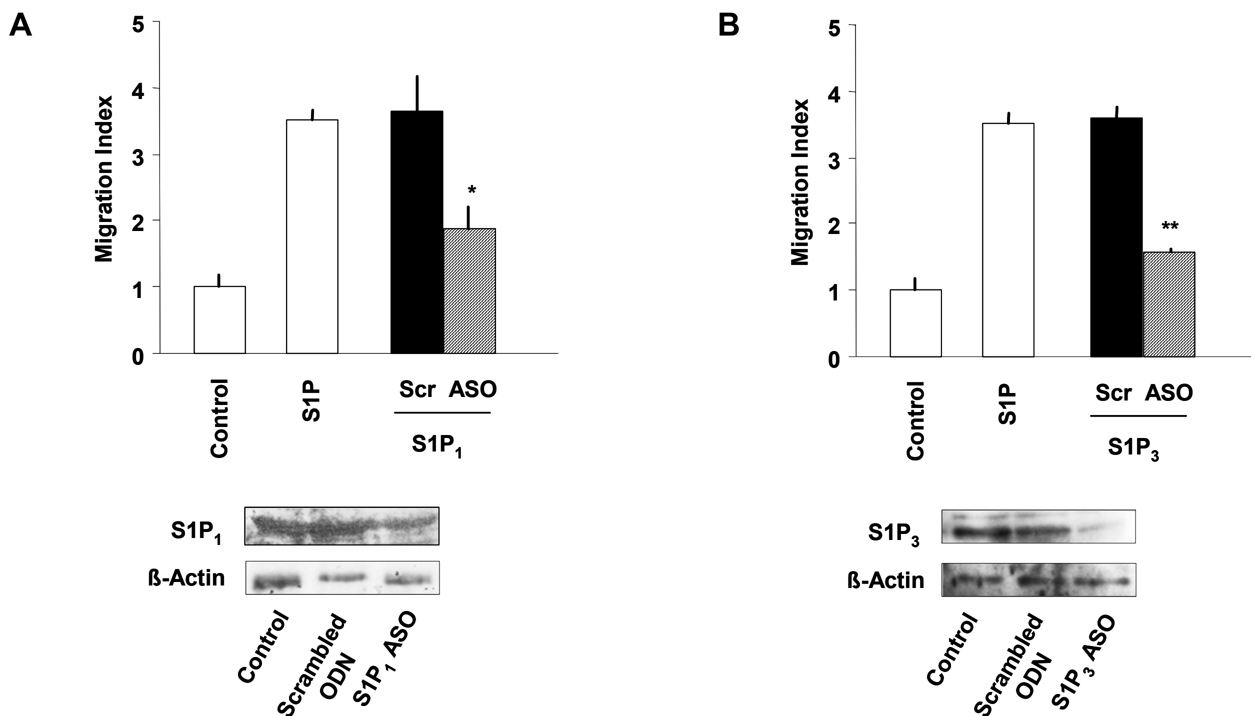


Fig. 15 *Dispaired motility of fibroblasts towards S1P in the presence of PTX - Cells were pre-incubated with PTX (200 ng/ml, 2 h (white bars)) and then, a migration assay was performed in the presence of control vehicle (0.4% BSA/PBS) or S1P 10^{-6} M. The data are expressed as means ± SD of duplicate determinations. Relative values of migration normalized using the migratory value in absence of stimuli are represented as MI. Similar results were obtained in two independent experiments. **, $p \leq 0.005$.*

Once the migratory activity of extracellularly added S1P through its receptors had been determined, the next step was to investigate which of the five known S1PRs are involved. For this purpose, the antisense technique against each of the expressed receptors was deployed. Transfection of the cells with ASO, and the corresponding scrambled ODNs, and western blot analysis using anti-$S1P_{1-5}$-specific antibodies revealed a significant reduction of receptor expression after exposure to specific antisense (upper blots). As loading control, immunoblotting was performed using anti-β-actin antibodies (lower blots). Because the decrease in mRNA expression of every single receptor was achieved by this treatment, the migratory activity of S1P was then investigated. As shown in Fig. 16.A and B, ASO directed against $S1P_1$ and $S1P_3$ inhibited the S1P-induced migratory response by almost 70%. Thus, confirming the involvement of these receptors in the chemotactic response of primary fibroblasts after S1P stimulation.



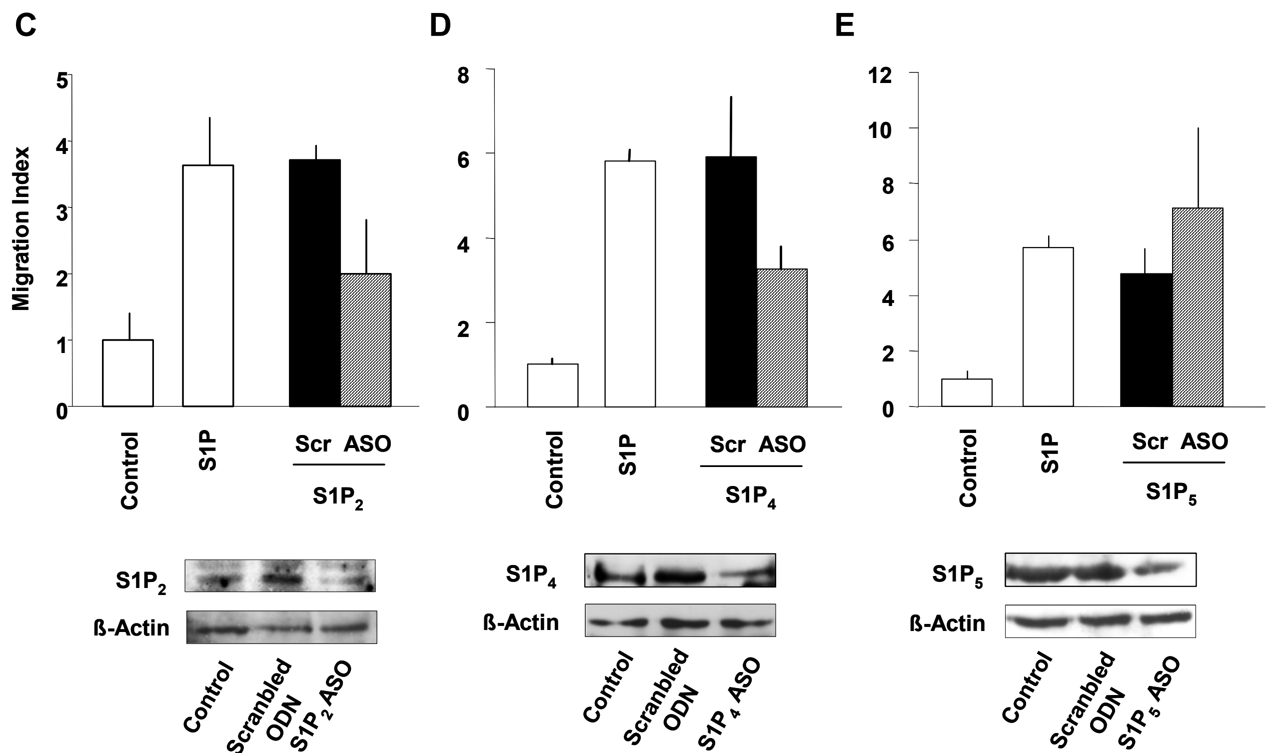


Fig. 16 *Involvement of S1PRs in the chemotactic activity of fibroblasts upon S1P - Primary cultures of human fibroblasts were transfected with $S1P_1$ (A)-, $S1P_3$ (B)-, $S1P_2$ (C)-, $S1P_4$ (D)- or $S1P_5$ (E)-specific ASO (striped bars) or control scrambled-ODNs (black bars). Western blot analysis using anti-$S1P_{1\text{-}5}$-specific antibodies served to confirm the reduction of receptor expression after exposure to specific antisense (upper blots), while anti-β-actin antibodies were used as control for loading (lower blots). Migration assays were then performed in the presence of control vehicle (0.4% BSA/PBS) or 10^{-7} M S1P (white bars). The data are expressed as MI and are means ± SD of duplicate determinations. *, $p \le 0.05$; **, $p \le 0.005$.*

Knocking-down $S1P_2$ (Fig. 16.C) and $S1P_4$ (Fig. 16.D) showed a tendency to reduce the number of migrated cells, but not in a significant way. After $S1P_5$-ASO-treatment (Fig. 16.E), no change in migration was observed indicating that neither $S1P_5$, nor $S1P_2$ and $S1P_4$, contributed to the migratory response of human fibroblasts. Transfection of dermal fibroblasts with the corresponding scrambled-ODN had no effect on the migratory response upon the agonist. Moreover, in its presence, S1P potently induced primary human fibroblasts motility in a similar extension as the untransfected cells.

3.2. Molecular pathways involved

3.2.1. The Smad system

3.2.1.1. Role of S1P on Smad proteins activation

S1P induced reciprocal responses on some cellular responses according to cell type and concentration. Regarding motility, S1P effects are both positive and negative depending on the receptors expressed and G proteins activated. These controversial effects are not unique for S1P; TGF-β has indeed analogue bimodal actions. Based on the similarity between the actions of S1P and TGF-β and the requirement for Smad3 to mediate the chemotactic properties of TGF-β, it was consequently investigated whether Smad3 might also be a mediator of S1P actions on fibroblasts. In order to evaluate the specificity of the Smad system in the S1P signaling pathway, S1P-induced Smad activation was analysed. Moreover, as complex formation between phosphorylated Smad3 and co-Smad4 is necessary for Smads nuclear import and subsequent binding to promoters of Smad3-responsive genes, the effect of S1P on this necessary Smad heteromerization was further investigated. Immunoprecipitation of stimulated fibroblast lysates with anti-Smad1/2/3 antibodies was carried out followed by electrophoresis. As only phosphorylated Smad2/3 can bind Smad4, immunoblotting for Smad4 demonstrated the transient Smad2/3-Smad4 complex formation in response to S1P. S1P stimulation of dermal fibroblasts caused an activation of the Smad2/3 after 15 min although the maximal effect occured at a concentration of 10^{-6} and 10^{-7} M after 30 min stimulation (Fig. 17, middle panel). In the absence of ligand, Smad2 and 3 were weakly phosphorylated. Moreover, determination of Smad4 on the cell lysate after precipitation of the activated complex, demonstrated the association of Smad3 with the Co-Smad4 after 30 min stimulation (Fig. 17, lower panel). Cytoplasmatic not-bonded Smad4 was found at 10^{-6} and 10^{-7} M S1P concentrations, corresponding to a significant enhancement of Smad3-Smad4 heteromerization. In order to control that the basal cytoplasmatic quantity of Smad4 was not altered after exposure to S1P, Smad4 in the whole lysate was firstly detected (Fig. 17, upper panel). A clear activation of R-Smads in response to S1P was observed, an effect that involved R-Smads phosphorylation and

heteromerization with Co-Smad4 finally leading to a functional complex, which is able to translocate in nucleus and evoke transcriptional activation of reporter genes.

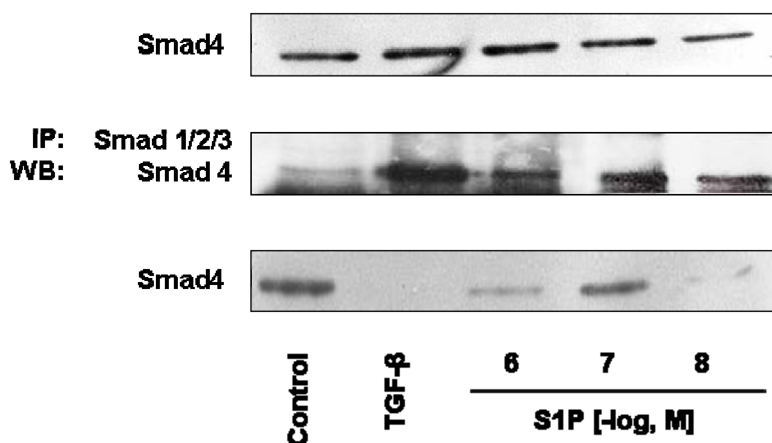


Fig. 17 Promotion of Smad3-Smad4 complexation upon treatment with S1P - Fibroblasts were treated with control vehicle (0.4% BSA/PBS), TGF-β (1 ng/ml) or different concentrations of S1P for 30 min. Probes of whole lysates were blotted with an anti-Smad4 antibody (upper panel). Lysates were afterwards immunoprecipitated with an anti-Smad1/2/3 antibody and Smad complexes were electrophoresed followed by immunoblotting with an anti-Smad4 antibody (middle panel). After immunoprecipitation, remaining lysates were again blotted with an anti-Smad4 antibody to detect free cytoplasmatic protein (lower panel).

3.2.1.2. S1P stimulates Smad nuclear translocation

It is well known that TGF-β induces Smads activation and their translocation to the nucleus, where they act as transcription factors. After finding out that, Smad proteins were required for S1P-signaling, the intracellular localization of Smad after activation was further investigated. To determine nuclear accumulation of Smad2/3, an immunofluoresecence analysis was carried out. Fibroblasts were stimulated with both S1P and TGF-β, fixed, and stained against Smad1/2/3. In agreement with other reports (Runyan et al., 2005), Smad2/3 entirely accumulated in the nucleus in response to TGF-β, whereas in basal conditions, Smads showed a diffused distribution throughout cytoplasma. Compared to TGF-β, S1P exhibited the distinct punctuate staining pattern associated with the nuclear accumulation of activated Smad transcription factors (Fig. 18).

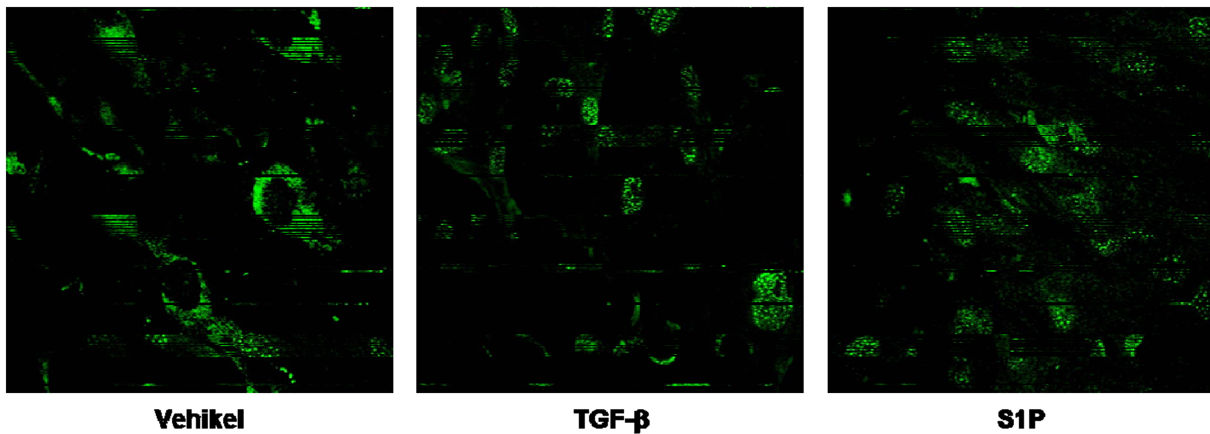


Fig. 18 *Smad nuclear translocation – Cells were stimulated with either vehicle (0.4% BSA/PBS), TGF-β (1 ng/ml) or S1P (10^{-7} M) for 30 min followed by fixation and staining with anti-Smad1/2/3 antibody. Images show Smads nuclear translocation upon activation, whereas under basal conditions, is their localization cytoplasmatic.*

3.2.1.3. Determination of S1PR participation in the activation of Smad proteins

As R-Smads activation appeared to be potentially important in fibroblasts function, the mechanisms underlying these observations were further focused.

The results in primary fibroblasts revealed the participation of $S1P_1$ and $S1P_3$ in the migratory response to S1P in a PTX-sensitive manner. Therefore, an aspect of interest was to examine which of the S1PRs might be involved in the Smad activation of human fibroblasts upon stimulation with their agonist S1P. Transfection of the corresponding receptor antisense ODN, which significantly reduced the expression of the receptors (Fig. 16, western blot), allowed observing the influence on Smad activation of each receptor by itself. Immunoprecipitation of cell lysates after S1P stimulation, as already described, revealed a Smad3-Smad4 complex. Thus, the absence of both $S1P_1$ and $S1P_3$ significantly diminished Smad activation after 30 min stimulation with a concentration of 10^{-7} M S1P (Fig. 19.A middle panel). To confirm the failure of heteromerization when switching off the expression of both $S1P_1$ and $S1P_3$ receptors, Smad4 in the lysate after immunoprecipitation of the activated complex was also detected. As expected, a higher level of free cytoplasmic Smad4 was seen when $S1P_1$ and $S1P_3$ mRNA expression was blocked (Fig. 19.A lower panel), corresponding to the decrease on Smad heteromerization and giving a role to $S1P_1$ and $S1P_3$ as effectors of Smad activation. As expected, ODN transfection did

not show any effect on cells, a fact seen by the absence of significant changes in Smad activation when the ASO-corresponding scrambled-ODN were transfected.

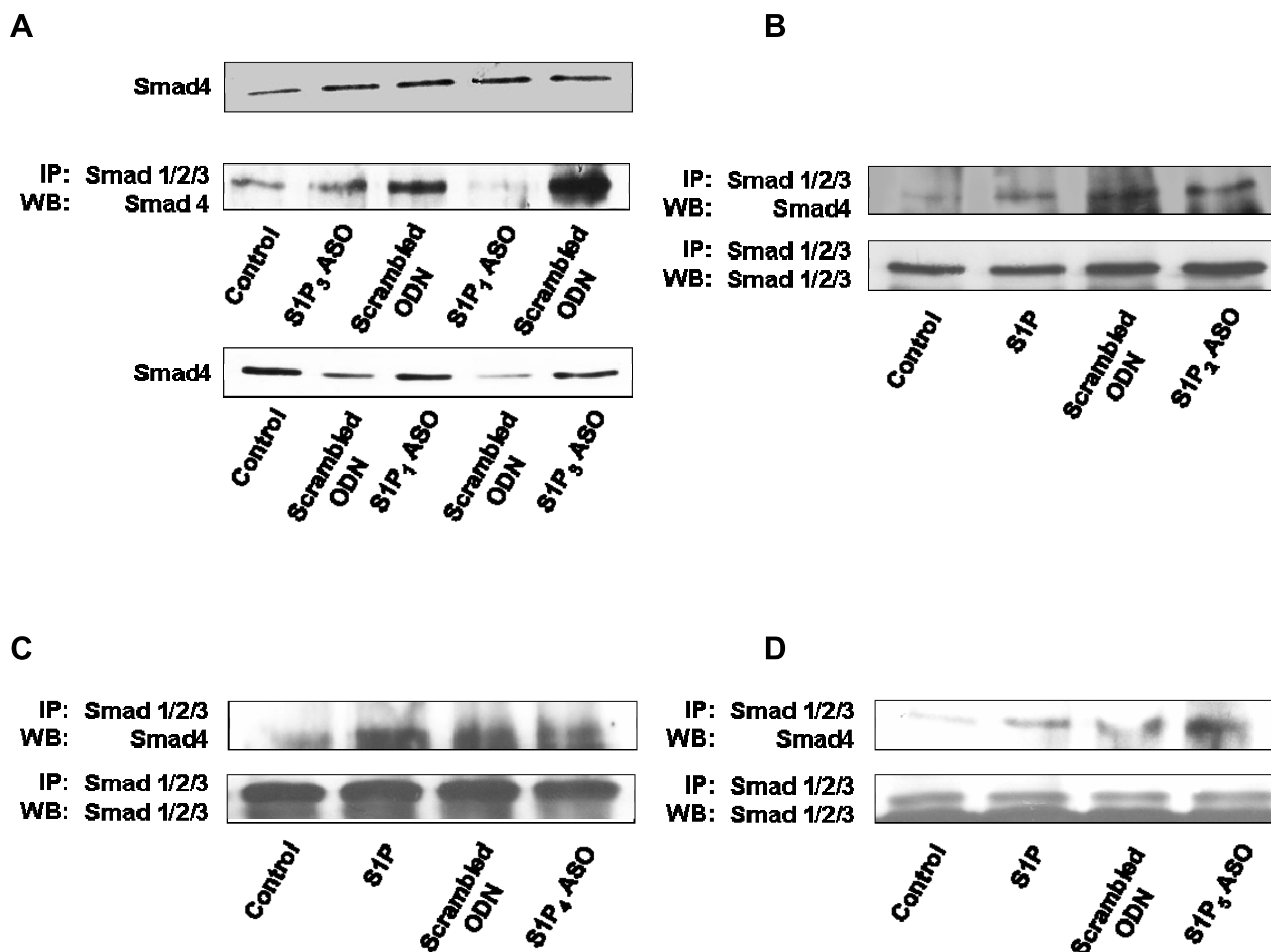


Fig. 19 *Involvement of $S1P_1$ and $S1P_3$ receptors on Smad3-Smad4 heteromerization - Fibroblasts were transfected with $S1P_1$ and $S1P_3$- (A), $S1P_2$- (B), $S1P_4$- (C) and $S1P_5$- (D)-specific ASO or control scrambled ODNs. Stimulation with S1P 10^{-7} M or vehicle (0.4% BSA/PBS) and immunoprecipitation followed by western blot analyse were performed as described. (A) Lysates were blotted with anti-Smad4 antibody form the whole lysate (upper blot), after immunoprecipitation of the whole lysate (middle blot) or from the lysate after immunoprecipitation (lower blot). (B, C, D) Lysates were immunoprecipitated with anti-Smad1/2/3 followed by western blotting with anti-Smad4 (upper blot) or anti-Smad1/2/3 (lower blot).*

Following the same procedures, the effects of $S1P_2$, $S1P_4$, and $S1P_5$ on the Smad activation were further investigated. Blocking the expression of the $S1P_2$ (Fig. 19.B) and $S1P_4$ (Fig. 19.C) did not affect the Smad3-Smad4 heteromerization. Interestingly, knocking-down $S1P_5$ expression (Fig. 19.D) displayed an increase in Smad activation, indicating that $S1P_5$ might also have a negative influence on the Smad

signal activation. These results indicate no decisive role for $S1P_2$, $S1P_4$, and $S1P_5$ in Smad activation upon S1P stimulation; however clearly confirm a crucial role for the $S1P_1$ and $S1P_3$ receptor for both S1P-mediated migration and Smad3 phosphorylation on human primary fibroblasts.
To give more evidences for the role of S1P receptors in the actions towards S1P, the effect of PTX incubation on S1P-induced Smad activation was in the following explored. Pre-incubation of human fibroblasts with PTX interfered with the ability of S1P to activate R-Smads, indicating that, as well as the chemotaxis, the activation event is a consequence of S1P receptor stimulation and, moreover confirmed the specific role of S1P in Smad3 activation.

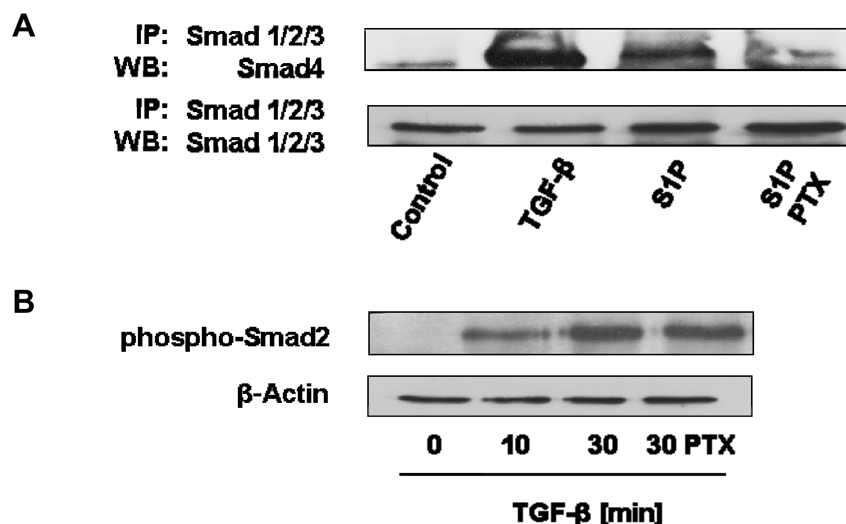


Fig. 20 *Influence of the Gi subunit on Smad activation - Cells were firstly incubated with PTX (200 ng/ml) for 2 h. (A) Lysates from stimulated cells (control vehicle (0.4% BSA/PBS), TGF-β 1 ng/ml, and S1P 10^{-8} M for 30 min) were immunoprecipated with anti-Smad1/2/3 antibody and blotted with anti-Smad4 (upper panel) or anti-Smad1/2/3 antibodies (lower panel). (B) The whole lysates from stimulated cells (vehicle and TGF-β 1 ng/ml for the indicated periods of time) were blotted with phospho-Smad2 antibody (upper panel) or with β-actin antibody (lower panel).*

As seen in Fig. 20.A, the heteromerization of R-Smad and Smad4 in response to exogenously added S1P was sensitive to PTX, confirming the involvement of Gi-coupled S1PRs. As expected, PTX had no effect on Smad activation induced by TGF-β (Fig. 20.B).

3.2.1.4. Abrogation of the Smad3 pathway: significance on S1P motility

These results clearly demonstrate the activation of Smad3 by S1P, but do not indicate an essential role of this signaling pathway on the biological responses. To evaluate the specific involvement of Smads in the S1P signaling pathway, fibroblast migration in the absence of Smad3 was examined. Therefore, mice Smad3 KO fibroblasts that lack the gene expressing the protein in question were cultivated and assessed. Interestingly, the basal migration rate of those cells was augmented. Although the differences between KO and WT motility were not significant, it became increased in the absence of stimuli. This mechanism of action could be attributed to a negative feed back caused by the lack of Smad3 protein (Fig. 21). In analogy to cells of human origin, S1P caused on murine WT fibroblasts a bell-shaped migration curve, whereas cultured Smad3 KO exhibited a reduced chemotaxis in a concentration-dependent manner (Fig. 21). In detail, KO cells stimulated with S1P concentrations from 10^{-6} to 10^{-9} M displayed impaired motility or did not exhibit any significant differences, but low doses of S1P (10^{-10} - 10^{-12} M) provoked an increase in migration. KO fibroblasts stimulated with low doses of S1P appeared to be effective in promoting cell movement despite lacking the Smad3 protein. Remembering the results on human fibroblasts, S1P 10^{-10} and 10^{-12} M induced chemokinesis. In order to elucidate whether the migratory response to low doses of S1P was due to a random movement and no longer to a chemotactic effect of S1P, the concentration gradient was disrupted and a chemokinesis assay was performed. Indeed, adding low doses of S1P to the upper and lower chamber induced a random movement of Smad3 KO fibroblasts rather than a directed migration (Fig. 22).

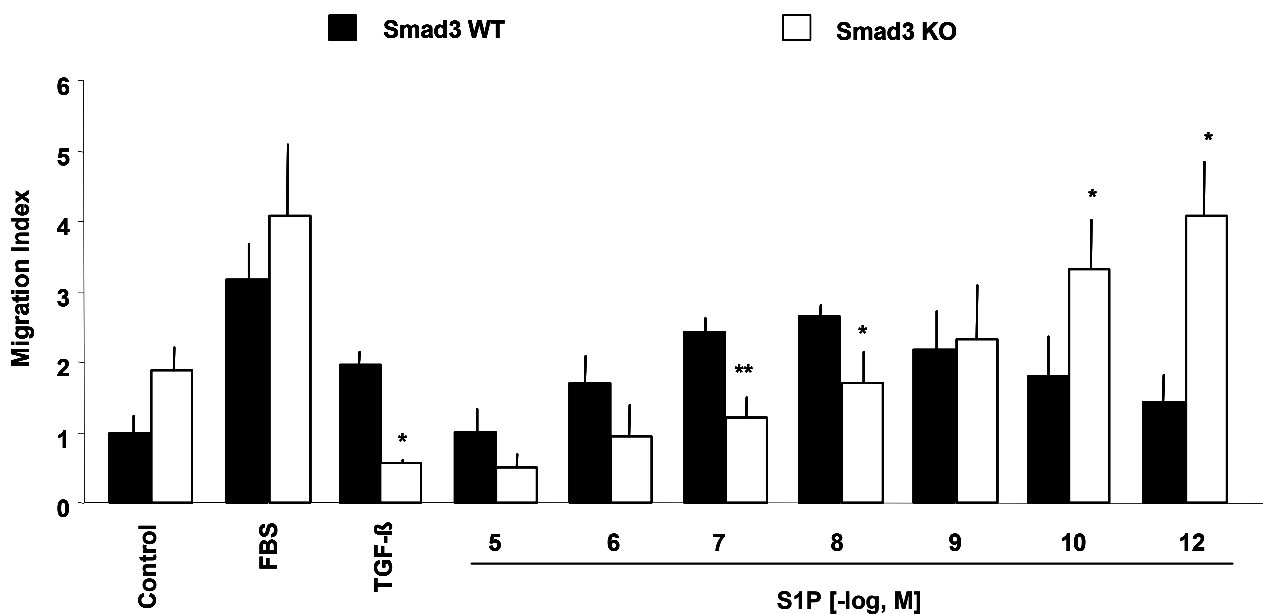


Fig. 21 *Motility of WT and Smad3 KO fibroblasts in response to S1P - Migration of murine cells was measured in response to vehicle (0.4% BSA/PBS), FBS (10%), TGF-β (1 ng/ml), and different gradients of S1P in a chemotaxis chamber as described. Relative values of migration normalized using the migratory value of WT cells in absence of stimuli are represented as MI of representative experiments. Each was repeated at least two times with similar results. The data are expressed as means ± SD of duplicate determinations. *, $p \leq 0.05$; **, $p \leq 0.005$.*

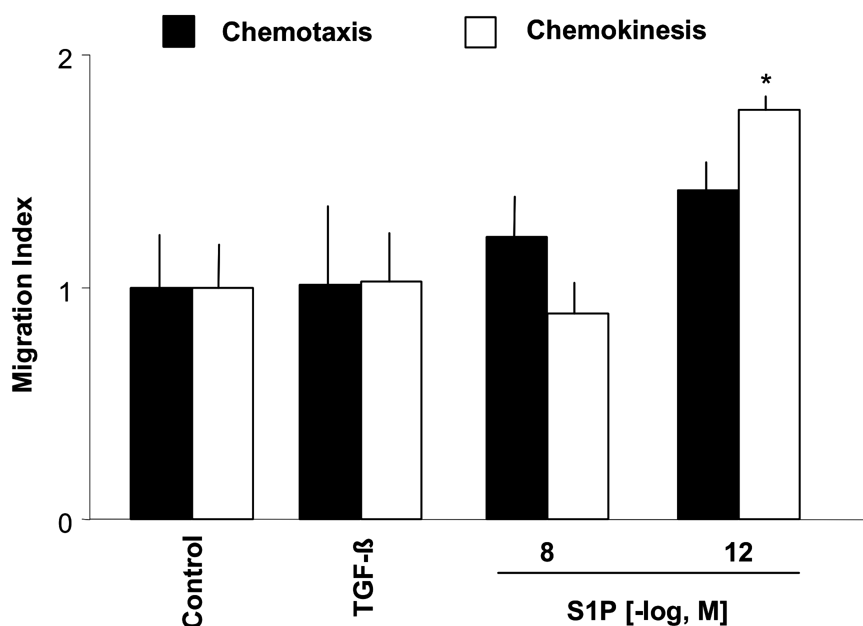


Fig. 22 *Chemokinesis of Smad3 KO fibroblasts in response to S1P - For chemokinetic actions, cells were treated with equal concentrations of TGF-β (1 ng/ml) and S1P on both sites of the filter (white bars) and the effect was compared to that of the chemotaxis (black bars). Relative values of migration normalized using the chemotactic value in absence of stimuli are represented as MI. The data are expressed as means ± SD of duplicate determinations. Similar results were obtained in two independent experiments. *, $p \leq 0.05$.*

The hypothesis that the migratory activity of Smad3 KO fibroblasts to low S1P concentrations (10^{-10} -10^{-12} M) was due to a chemokinetic effect was in that way confirmed. Thus, cellular response to S1P might depend on its concentration.

It is well known that cells lacking Smad3 do not migrate upon TGF-β. As expected, murine KO fibroblasts also failed to migrated after TGF-β stimulation (Fig. 21). Furthermore, the chemokinetic effect induced by high concentrations of TGF-β (1 ng/ml) on KO cells was slightly increased compared to the basal state, but still dependent on the presence of the protein. In conclusion, motility of human fibroblasts upon TGF-β was Smad3 sensitive, while the migratory effects of S1P depended on its concentration. Higher S1P doses (10^{-6} - 10^{-9} M) evoked chemotaxis in a Smad-dependent way, whereas the lower ones (10^{-10} – 10^{-12} M) promoted a chemokinetic response that might be regulated through different mechanisms of action than the Smad system. These results confirmed the essential role of Smad3 as downstream signal transducer for both molecules.

3.2.2. The MAPK cascade

3.2.2.1. Phophorylation of the MAPK ERK1/2 in response to S1P

It is well known that the Smad proteins can be phosphorylated upon TGF-β stimulation and alternatively by the MAPK system. Hereby, it is also revealed that the lipid mediator S1P had also been able to activate the R-Smads. Whether this effect was directly or indirecly evoked remained unclear. Since S1P can stimulate MAPK activity in different cells types (Hsieh et al., 2006; Xin et al., 2004) (Donati et al., 2005) (Cuvillier et al., 1996), it was of great interest to examine if the MAPK p44/p42 (ERK1/2) were phosphorylated after S1P stimulation in human dermal fibroblasts and thereby phosphorylating the Smads. Activation of these kinases was assayed in a western blot by use of an specific antibody for the phosphorylated, active forms of p44/p42 MAPKs. As shown in Fig. 23, S1P stimulated a time- and concentration-dependent phosphorylation of p44/p42 MAPK in human fibroblasts. Compared to the basal level, the maximal response to S1P was obtained within 10 min (Fig. 23.A) at a concentration range of 10^{-7} – 10^{-9} M (Fig. 23. B). There was no change at the level of endogenous p44 MAPK as determined using p44 MAPK antibody. As in other cell types, S1P was able to transient phosphorylate the MAPK

ERK1/2. With respect to the maximum S1P effect in ERK phosphorylation (peaking after 10 min) and Smad activation (30min), an indirect phosphorylation of Smads by S1P could not be excluded.

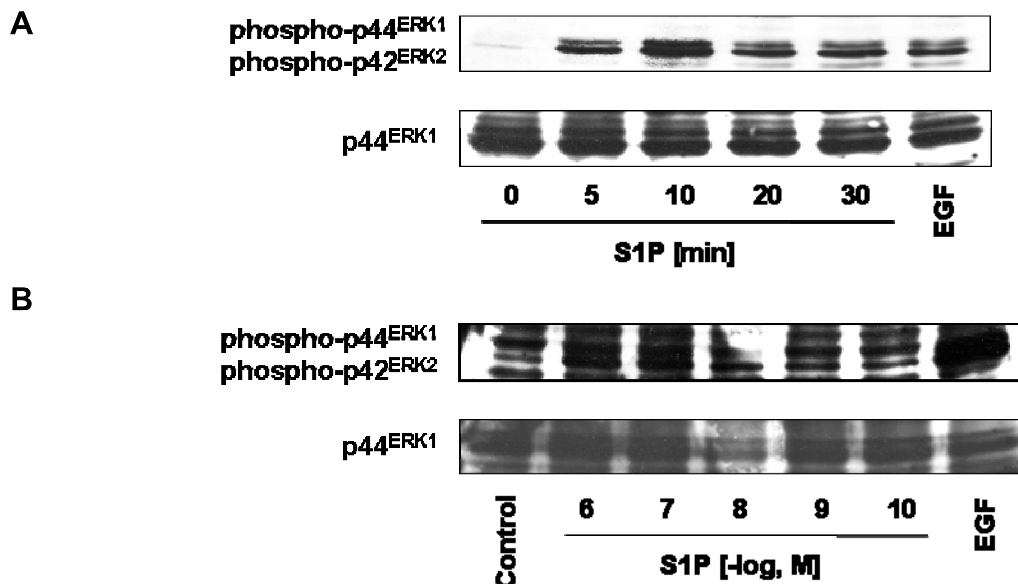


Fig. 23 S1P-induced phosphorylation of ERK1/2 MAPK - Fibroblasts were stimulated with control vehicle (0.4% BSA/PBS), EGF (1 ng/ml), and S1P 10^{-8} M for the indicated times (A) or for 10 min with the indicated concentrations (B). Lysates were electrophoresed and immunoblotted with anti-phopho-ERK1/2 or with anti-ERK1 antibodies as loading control.

3.2.2.2. Role of ERK1/2 activation in the migratory effect of S1P

Cell migration is often connected with or rather dependent on the activation of ERK1/2 (Nickl-Jockschat et al., 2007). The underlying mechanism, however, are very complex and not completely clarified yet. In order to examine if ERK1/2 is likewise involved in the migratory response of fibroblasts a chemotaxis assay was performed in the presence of PD098059, a specific inhibitor of the ERK activator kinase MEK (Dudley et al., 1995). It has further been shown in this study (Fig. 12 and Fig. 22) that low concentration of S1P were unable to induce chemotaxis but rather chemokinesis in a Smad independent manner, while higher doses caused chemotaxis dependent on the Smad3 protein. Therefore, murine Smad3 WT and KO fibroblasts were used for these experiments. As shown in Fig. 24, WT cells migrated normally to S1P and

its motiltity was unaffected after disruption of ERK1/2 activation. As already described (Fig. 21), KO cells failed to migrate towards S1P 10^{-8} M but they still migrated to 10^{-12} M. Interestingly, this migratory effect was blocked after interfering with ERK1/2 activation (Fig. 24). These results provided evidences for a participation of ERK1/2 on the Smad3-independent chemokinesis induced by low doses of S1P.

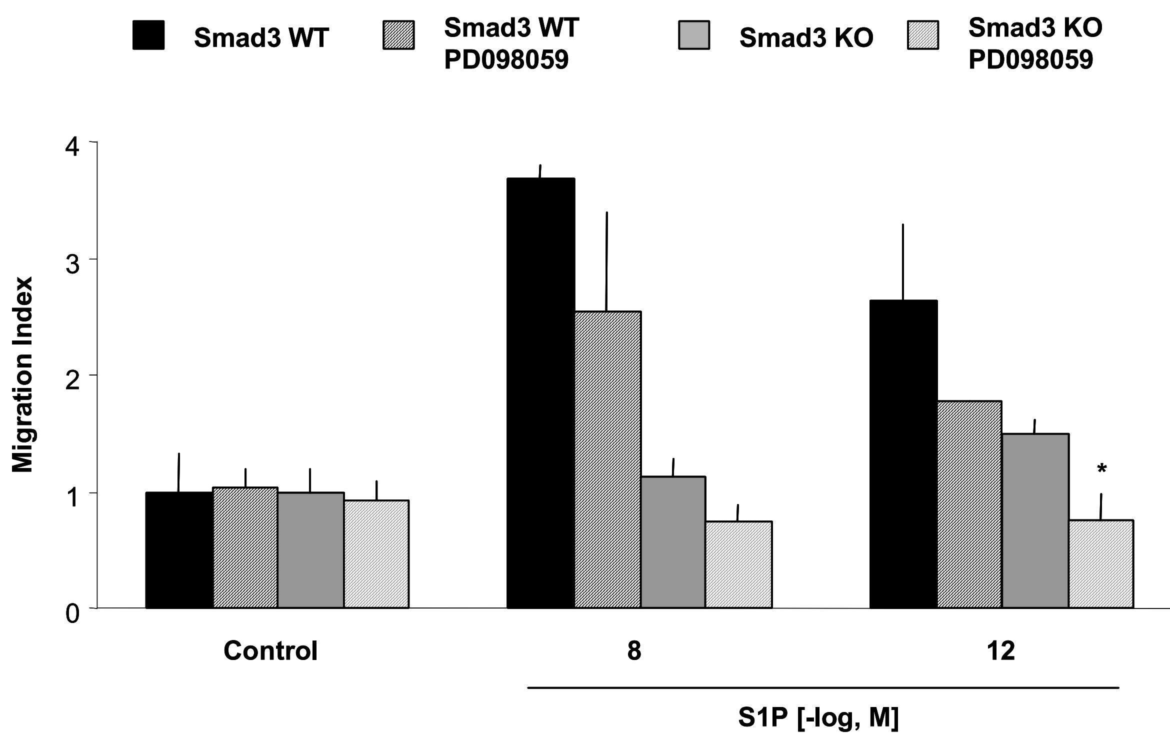


Fig. 24 *Involvement of the MAPK pathway in Smad3-independent chemokinesis – Cells were treated with the MEK inhibitor, PD098059 (50 µM), or vehicle (0.1% DMSO) for 1.5 h. Migration assay was then performed in WT and KO cells in the presence of control vehicle (0.4% BSA/PBS) and S1P at the indicated concentrations. Values are expressed as means ± SD of duplicate determinations. Relative values of migration normalized using the migratory value of WT or KO cells in absence of stimuli are represented as MI of representative experiments, each repeated at least two times with similar results. *, $p \leq 0.05$.*

3.3. Interplay of S1P and TGF-β signaling

3.3.1. Effects of TβR-I inhibition on S1P actions

Careful analysis of the biochemical fibroblast responses to S1P revealed remarkable and extensive similarities between S1P-mediated actions and the signaling pathways evoked by TGF-β. These results clearly demonstrate an activation of the TGF-β-signaling molecule, Smad3 by S1P through $S1P_1$ and $S1P_3$ and indicate an essential role of this signaling pathway for chemotaxis of human dermal primary fibroblasts, but the mechanism of action underlying these results remained unclear. Crosstalk between different transactivated signal pathways has been well described as a conserved mechanism of signal amplification that can be regulated at receptor level. Transactivation of the chemotactic S1P receptor, $S1P_1$, with PDGF and VEGF served as example for signal amplification (Igarashi et al., 2003) (Waters et al., 2006). According to this, it would be possible that S1P might require the TβRs and their effectors, Smad proteins, to evoke its signal. For that purpose, SB431542, a competitive ATP binding site kinase inhibitor of the TβR-I kinase (Inman et al., 2002) (Laping et al., 2002), was applied in a chemotaxis assay. Indeed, SB431542 at a concentration of 10 µM, significantly reduced TGF-β motility and, most interestingly, had similar effects on S1P-induced motility, whereas serum-induced chemotaxis was not significantly influenced (Fig. 25). Abrogation of TβR-I phosphorylation and subsequent receptor activation diminished S1P-induced motility up to 50% and induced a failed response of its agonist TGF-β, which response reached basal levels. Once the influence of the TβR-I in the S1P-induced motility of human fibroblasts was assessed, a question of matter was to determine its influence concerning chemotaxis and chemokinesis. Therefore, both processes were analysed in the presence of the TβR-I inhibitor, SB431542, as already described. As expected, blocking of TGF-β signaling interfered with the chemotaxis promoted by both, TGF-β and S1P. On the contrary, neither TGF-β- nor S1P-induced chemokinesis were affected after treatment with SB431542 (Fig. 26). These results clearly confirmed the participation of the TβR-I in the directed motility of human dermal primary fibroblasts caused by S1P, whilst left to a different mechanism of action the random movement of these cells.

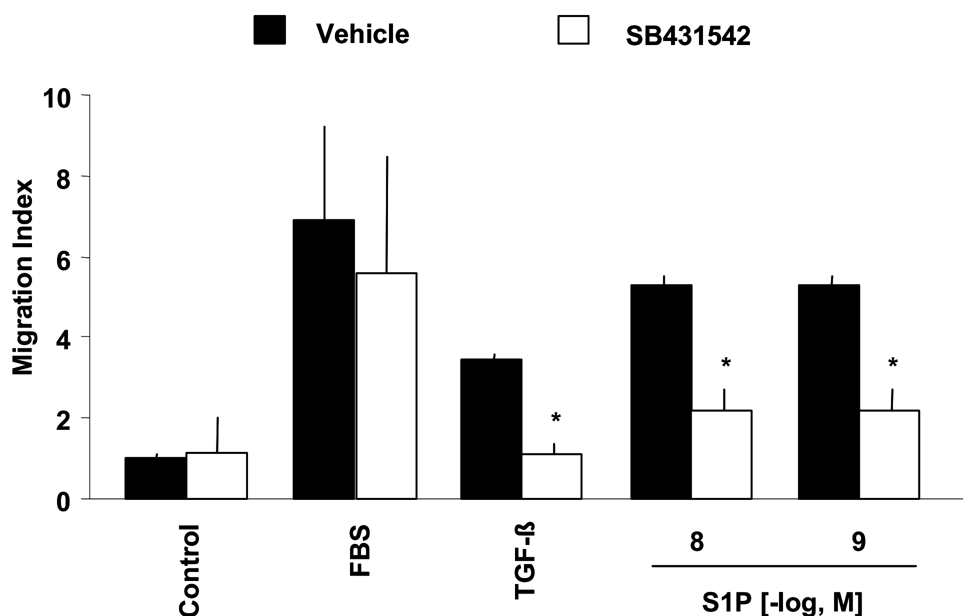


Fig. 25 *Migration of human fibroblasts in the presence of the TβR-I inhibitor – Cells were pre-incubated with 10 µM SB431542 or vehicle (0.1% DMSO) for 30 min previous to the assay. Migration was performed in response to vehicle (0.4% BSA/PBS), FBS (10%), TGF-β (0.1 ng/ml), and S1P at the indicated concentrations. Values are expressed as means ± SD of duplicate determinations and represented as MI of representative experiments, each repeated at least two times with similar results. *, p ≤ 0.05.*

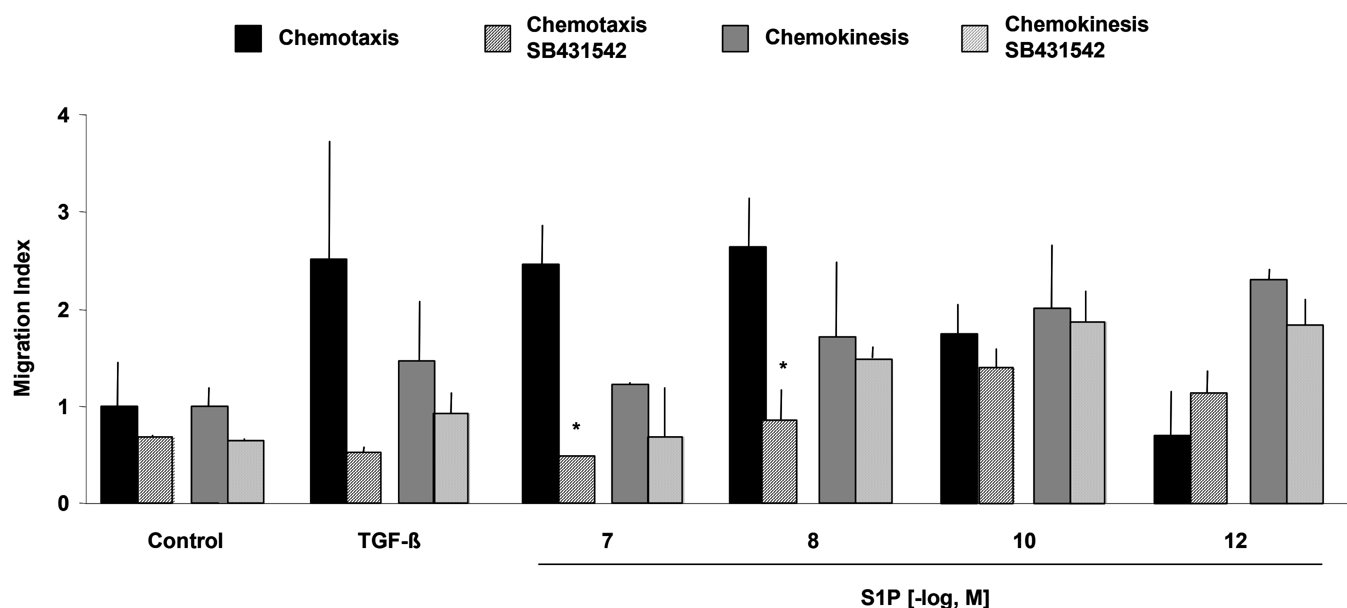


Fig. 26 *Chemotaxis and Chemokinesis of fibroblasts in the presence of the TβR-I inhibitor – Cells were incubated with 10 µM SB431542 or vehicle (0.1% DMSO) for 30 min before the assay was performed. Migration was assessed in a transwell assay in response to vehicle (0.4% BSA/PBS), TGF-β (0.1 ng/ml), and S1P at the indicated concentrations. Chemokinesis assay was performed as described, adding control vehicle, TGF-β or S1P to the upper and lower chamber. Values are expressed as means ± SD of duplicate determinations. Relative values of migration normalized using the chemotactic or chemokinetic values of cells in absence of stimuli was represented as MI of representative experiments, each repeated at least two times with similar results. *, p ≤ 0.05.*

To further substantiate whether the typical TGF-β signaling pathway involving Smad proteins are also involved in S1P-mediated actions, Smad activation was measured in the presence of SB431542. Consistent with the migration experiments, Smad activation in response to S1P and TGF-β was markedly reduced when TβR-I kinase was inhibited (Fig. 27). These experiments suggest a complex interplay between both S1P and TβR-I receptors to affect S1P-mediated Smad3 activation and chemotaxis.

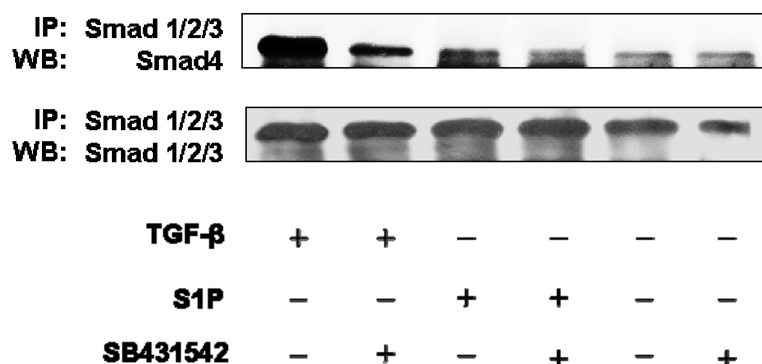


Fig. 27 Participation of TβR-I in Smad3 activation upon treatment with S1P - Fibroblasts were treated with 10 μM SB431542 or vehicle (0.1% DMSO) for 30 min followed by stimulation with control vehicle (0.4% BSA/PBS), TGF-β (1 ng/ml) or S1P 10^{-7} M for 30 min. Lysates were immunoprecipitated with anti-Smad1/2/3 antibody and Smad complexes were electrophoresed followed by immunoblotting with anti-Smad4 (upper blot) or anti-Smad1/2/3 antibody as a control for protein precipitation.

3.3.2. Agonist-induced $S1P_1$ internalization

The underlying mechanism of the interaction between S1P and TGF-β receptors was further investigated. Firstly, how S1P reacts after agonist binding was analysed and afterwards how both signals, S1P and TGF-β, might overlap. To determine whether S1P treatment could induce internalization of its receptor, the $S1P_1$-GFP plasmid was transfected into the 3T3 fibroblast cell line. Overexpression of $S1P_1$ in cells efficiently transfected was controlled by detection of a fluorescent signal emitted by GFP. As seen in Fig. 28, the treatment of 3T3 fibroblasts with S1P for 30 min, clearly induced agonist-dependent receptor internalization indicated by a release of receptor from the plasma membrane into the cytoplasm.

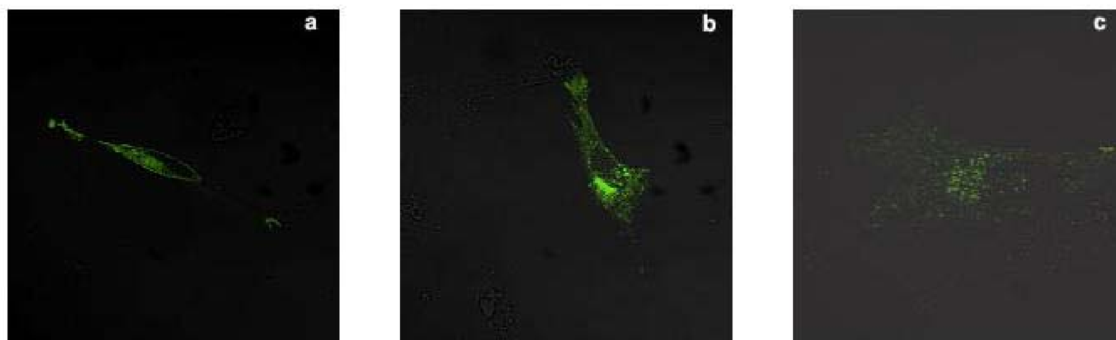


Fig. 28 $S1P_1$-GFP internalization toward S1P stimulation – 3T3 fibroblasts were grown on coverslips and then transfected with 1 µg plasmid for 24 h followed by stimulation with vehicle (0.4% BSA/PBS) (a) or S1P 10^{-6} M (b and c) for 30 min. $S1P_1$-GFP expression and internalization were visualized by confocal microscopy using a LSM 510 microscope.

3.3.3. S1P heterologous desensitization of TGF-β-signaling

The involvement of TβR-I in S1P-induced actions has previously been shown in such a way that blocking of the TGF-β signal clearly interfered with S1P signaling. As seen in chapter 1.1.2.1, ligand binding of GPCRs results in receptor phosphorylation, internalization, and subsequent signal desensitization. Confirming this characteristic of GPCRs, S1P stimulation of fibroblasts leaded to $S1P_1$ receptor internalization. Thus, a further aspect of interest was to analyse whether $S1P_1$ desensitization might interfere with the TGF-β signal, thereby confirming the requirement of the TGF-β pathway for S1P signaling. To this end, fibroblasts were firstly pre-treated with S1P to promote receptor-induced signaling. Then, chemotaxis and Smad phosphorylation upon TGF-β stimulation were measured. TGF-β induction of both chemotaxis (Fig. 29.A) and phosphorylation of the 465/467 serine residues of the R-Smad, Smad2 (Fig. 29.B), were diminished after pre-incubation of fibroblasts with S1P. Furthermore, consisting with the previous confocal images of agonist-dependent receptor internalization (Fig. 28.b and c), the chemotactic activity of fibroblasts towards S1P was also diminished after a second immediate stimulation. On the contrary, desensitization of S1P signaling did not affect serum-induced chemotaxis (Fig. 29.A).

Two important facts to understand the molecular ways involved in S1P signaling are provided here: $S1P_1$ undergoes agonist-dependent receptor internalization and S1P exhibits heterologous desensitization of the TGF-β signaling.

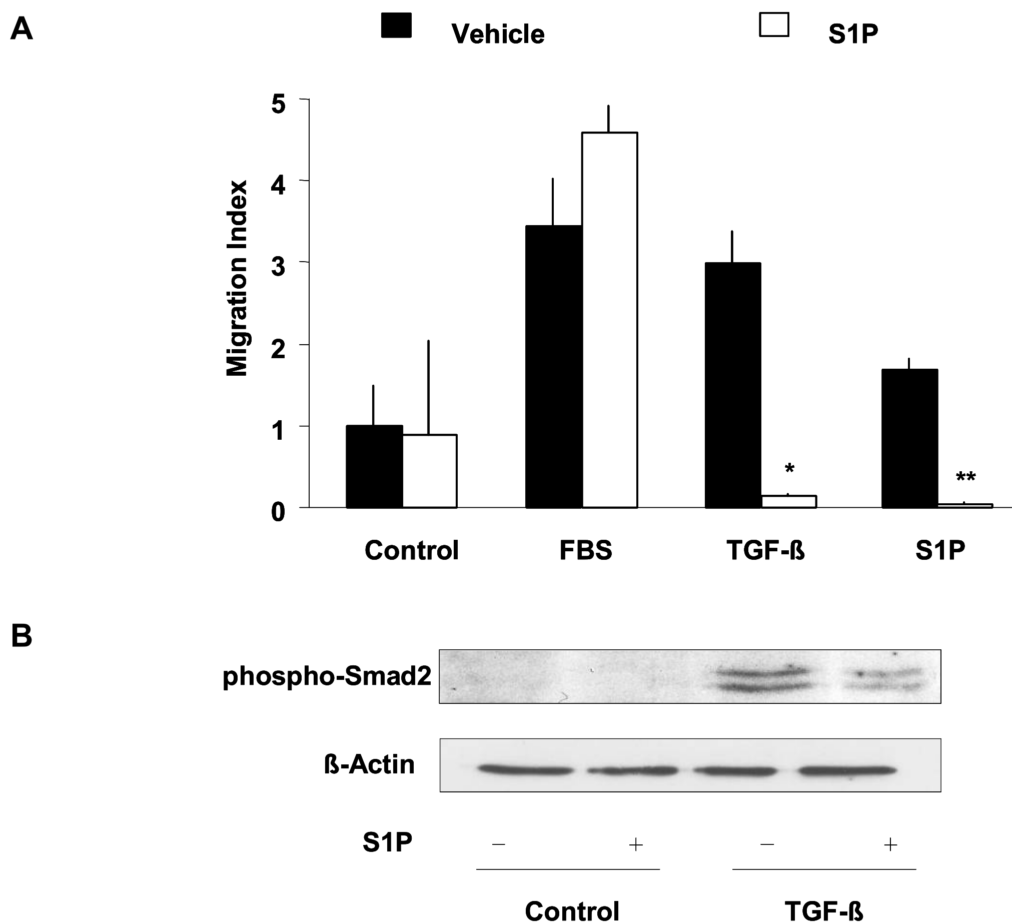


Fig. 29 *Influence of S1P receptor desensitization on TGF-β signaling – Migration assay (A) and Smad2 phosphorylation (B) were determined after 30 min pre-incubation of fibroblasts with 10^{-6} M S1P. (A) Migration was performed in a transwell assay in response to control vehicle (0.4% BSA/PBS), FBS (10%), TGF-β (0.1 ng/ml), and S1P (10^{-8} M). Values are expressed as means ± SD of duplicate determinations and represented as MI of representative experiments, each repeated at least two times with similar results. *, $p \leq 0.05$; **, $p \leq 0.005$. (B) Smad2 phosphorylation was detected from the whole lysate of stimulated fibroblasts using an antibody against phospho-Ser 465/467 or β-actin as loading control.*

Additionally, pre-incubation of fibroblasts with TGF-β (Fig. 30) caused any influence on the chemotaxis induced by S1P, indicating a specific effect of the lysophospholipid on TGF-β signaling. On the contrary, TGF-β stimulation of TGF-β-desensitized fibroblasts (Fig. 30) suggested a predisposition to reduce its migratory answer, though not significantly.

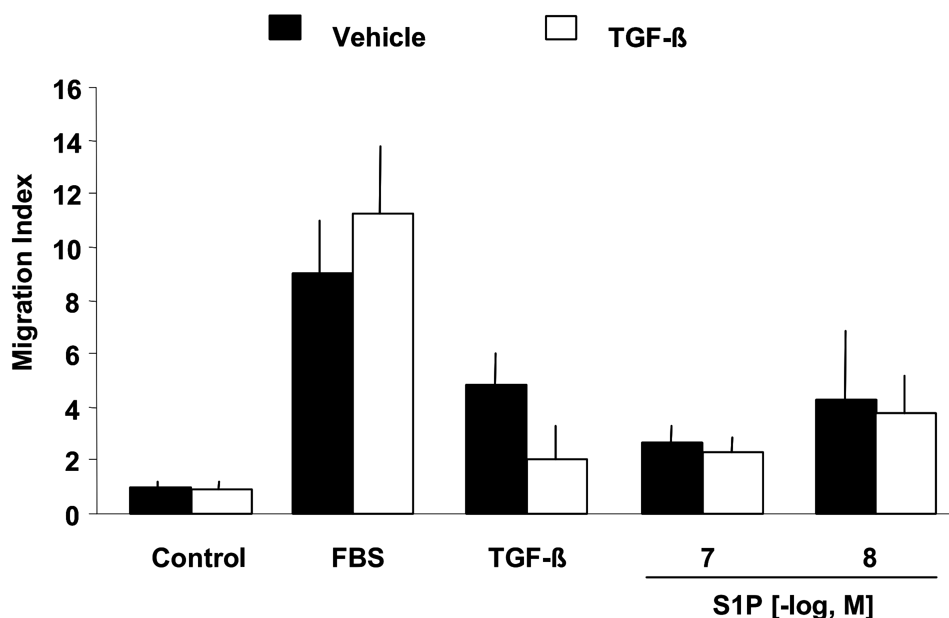


Fig. 30 *Migration of TGF-β pre-treated fibroblasts – Cells were stimulated 30 min with 1 ng/ml TGF-β or control vehicle before the assay. Migration was performed in a transwell assay in response to control vehicle (0.4% BSA/PBS), FBS (10%), TGF-β (0.1 ng/ml), and S1P at the indicated concentrations. Values are expressed as means ± SD of duplicate determinations and represented as MI of representative experiments, each repeated at least two times with similar results.*

3.3.4. Role of ERK1/2 in the desensitization of the TGF-β signal through S1P

A broad range of studies exists, describing that the crosstalk between Smads and MAPKs contribute to promote cellular responses, including motility (Watanabe et al., 2001) (Subauste et al., 2004) (Le Stunff et al., 2004) (Long et al., 2006) (Janda et al., 2006). To determine if ERK1/2 activation is essential for the repression of TGF-β signaling by S1P, fibroblasts were firstly treated with PD098059 and migration was then measured after S1P pre-incubation. As presented in Fig. 31, disruption of the MAPK cascade was unable to significantly reverse the inhibition of motility caused by

TGF-β stimulation of S1P-desensitized fibroblasts. These results give evidences that S1P signaling requires TβRs to activate Smads and promote motility, and exclude the possibility that the decrease of TGF-β response was an effect of Smad inactivation by MAPKs.

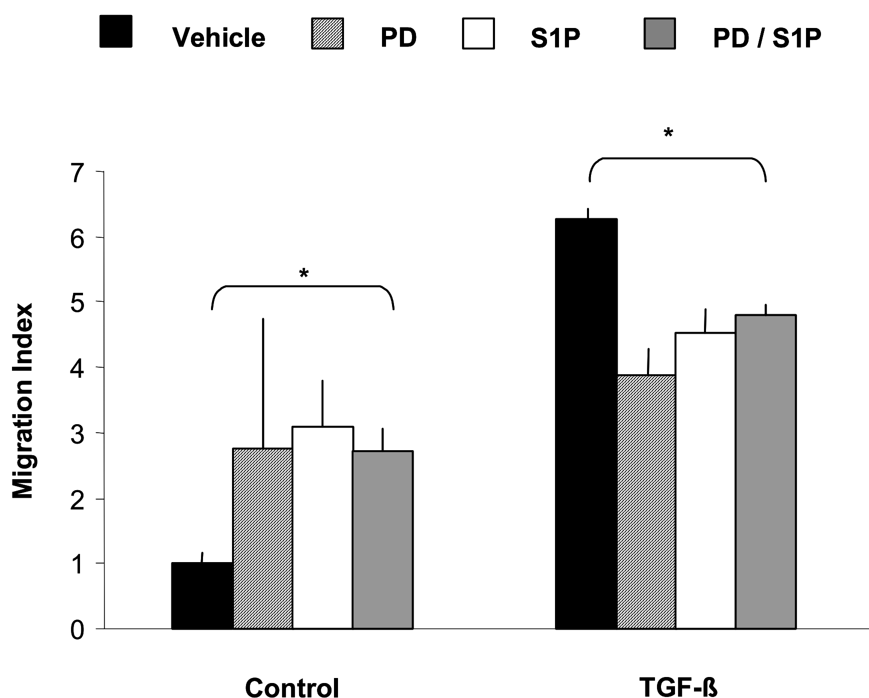


Fig. 31 *Motility of desensitized fibroblasts after treatment with PD098059 – Migration assay was determined in the presence of vehicle (filled bars) or after 1.5 h incubation with PD098059 (50 µM) (striped bars), pre-incubation with 10^{-6} M S1P (white bars) or both (grey bars). Migration was performed in a transwell assay in response to control vehicle (0.4% BSA/PBS) or TGF-β (0.1 ng/ml). Values are expressed as means ± SD of duplicate determinations and represented as MI of representative experiments, each repeated at least two times with similar results. *, $p \leq 0.05$.*

3.3.5. Transfection of fibroblasts with the HA-tagged TβR-I plasmid

To more rigorously prove a possible crosstalk between both the S1P and the TGF-β signaling pathway, human fibroblasts were transient transfected with a HA-tagged TβR-I (HA-TβR-I) plasmid and further experiments were carried out. Firstly, to optimize the transfection procedure, different quantities of HA-TβR-I plasmid were transfected for different periods of time. Western-Blot analysis of HA-TβR-I using an anti-HA-Probe antibody confirmed the expression of the protein in cells efficiently

transfected with the foreign DNA in a time and dose dependent manner. As seen in Fig. 32, transfection of 1 µg plasmid for 24 hours was enough to significantly force the expression of the new protein. The HA band was not present in the negative control group that included mock-transfected cells (in the absence of DNA).

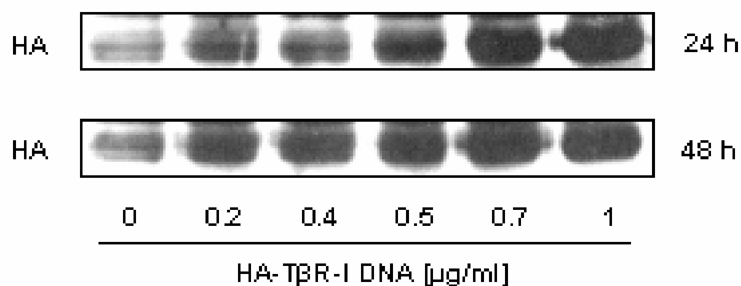


Fig. 32 HA-TβR-I transfection of fibroblasts – Cells were transfected with different concentrations of plasmid for different periods of time (24 and 48 h). Lysates containing the total amount of proteins were electrophoresed and the TβR-I was detected using an anti-HA-probe antibody.

3.3.6. Signal retrieval in cells overexpressing the TβR-I

The ability of the transfected plasmid to promote signal was subsequently determined. Human primary fibroblasts were therefore either mock or with the HA-TβR-I plasmid transfected and migration was performed in the presence of its agonist, TGF-β. Compared to mock cells, transfected fibroblasts migrated normally to the natural ligand (Fig. 33), indicating active TβRs that react to their agonist. In parallel, cells were pre-incubated with S1P after the tranfection of the construct, but before the assay was performed. S1P caused again heterologous desensitization of TGF-β signaling. Interestingly, motility upon TGF-β stimulation was partially restored in HA-TβR-I-transfected cells after S1P-induced desensitization. Thus, heterologous desensitization of TGF-β signaling by S1P could be restored when the TβR-I was overexpressed (Fig. 33).

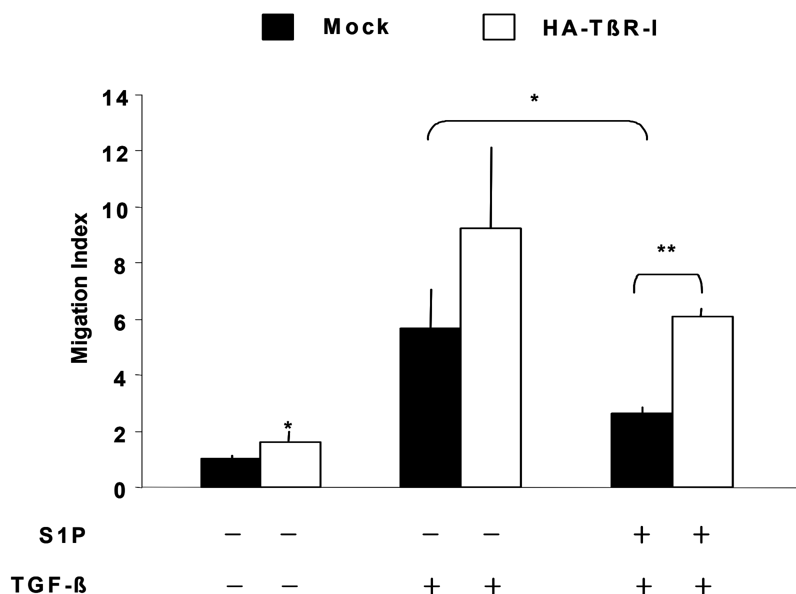


*Fig. 33 Restoration of the TGF-β signal on desensitized fibroblasts after TβR-I overexpression – HA-TβR-I (white bars) and mock-transfected (black bars) cells were allowed to migrate towards control vehicle (0.4% BSA/PBS) or TGF-β (0.1 ng/ml) for 4.5 h in a transwell assay. Some cells were previously pre-incubated with 10^{-6} M S1P. Values are expressed as means ± SD of duplicate determinations and represented as MI of representative experiments, each repeated at least two times with similar results. *, $p \leq 0.05$; **, $p \leq 0.005$.*

3.3.7. Down regulation of membrane TβRs after S1P stimulation

To further investigate the requirement of TβRs to promote S1P signaling, and considering the ability of S1P to induce its receptor internalization and the desensitization of TGF-ß signaling, a down-regulation of TßRs from the membrane after S1P stimulation could be expected. Western blot analysis allowed surveillance of the S1P-evoked TβR internalization. As both receptors, TβR-I and $S1P_1$, are located on the cell surface an isolation of the membrane fraction of the cells was carried out. To this purpose, fibroblasts were firstly transfected with the HA-TβR-I. Afterwards, membrane fractions of stimulated cells were isolated and subjected to western blot analysis using an antibody against HA-protein. In agreement with the results on Fig. 30, TβR-I significantly disappeared from the membrane after agonist stimulation. Most interestingly, treatment with S1P resulted in an even more enhanced effect than TGF-β itself did (Fig. 34 A). As expected, no significant

changes were observed when fibroblasts were only treated with vehicle and no HA protein was detected in the mock-transfected cells. To ensure that the decrease of TβR in the membrane fraction was due to the stimulation and not to a possible damage of the plasma membrane integrity, the same PVDF-membrane was stripped off its antibodies and subjected to a new immunoblotting process to detect β-catenin, a membrane protein which exclusively locates on the plasma membrane. Indeed, the membrane isolation process of HA-TβR-I-transfected cells was successful. Same amounts of β-catenin were found in the plasma membrane (Fig. 34 B) and hence, the decrease of TβR-I was specific to S1P treatment.

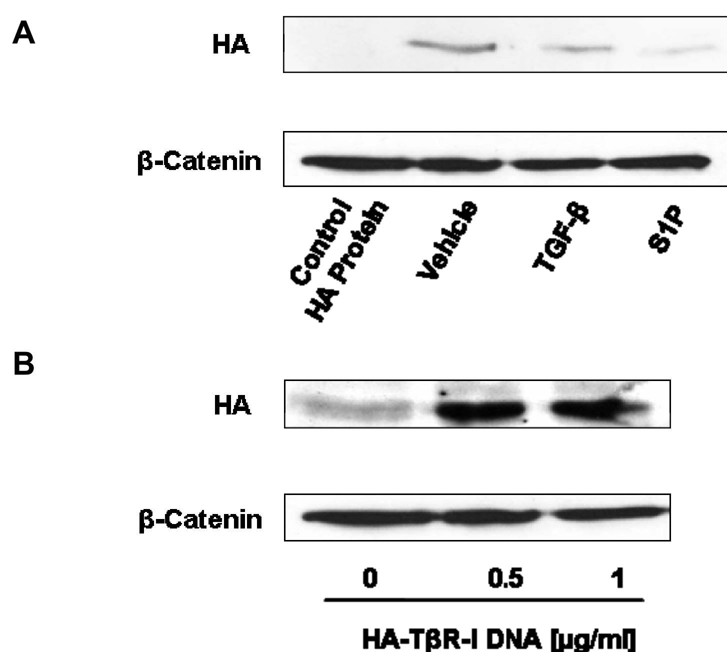


Fig. 34 *S1P-induced TβR-I down regulation from the plasma membrane – Cells were transfected with the HA tagged TβR-I (1 µg, 24 h) followed by stimulation with control vehicle (0.4% BSA/PBS), 1 ng/ml TGF-β or 10^{-6} M S1P (A) or with different plasmid quantities (B). Membrane protein purification was performed as described in Materials and Methods. The cold membrane fraction was then electrophoresed and immunoblotted with an anti-HA-probe antibody. Each corresponding PVDF membrane was then stripped off and blotted against β-Catenin as control for membrane integrity.*

3.3.8. Abrogation of S1P-induced internalization of TβR-I

TβRs endocytose through a clathrin-mediated mechanism to promote signaling (Penheiter et al., 2002). Depletion of intracellular potassium disrupts formation of the clathrin-coated pit (Larkin et al., 1986), thereby inhibiting TβR internalization (Lu et al., 2002b). To determine whether endocytosis was required for S1P transactivation of TβR-I, cell fractioning experiments followed by membrane purification under conditions of cellular potassium depletion to inhibit clathrin-coated pit formation were performed. Under normal conditions of intracellular potassium, both S1P- and TGF-β-induced TβR-I membrane down-regulation was again observed. When Fibroblasts were treated with an hypotonic medium followed by two isotonic potassium-free buffers to deplete intracellular potassium and inhibit endocytosis, the levels of membrane TβR-I accumulation after S1P and TGF-β exposure compared with that of potassium control conditions were restored (Fig. 35). Thus, potassium depletion significantly inhibited TβR-I endocytosis; however, ligand-mediated receptor endocytosis was restored when 10 mM KCl was included in the buffer. These results clearly indicate a clathrin-mediated endocytosis of TβR-I upon TGF-β and connotatively give a novel role for S1P as mediator of TβR-I endocytosis.

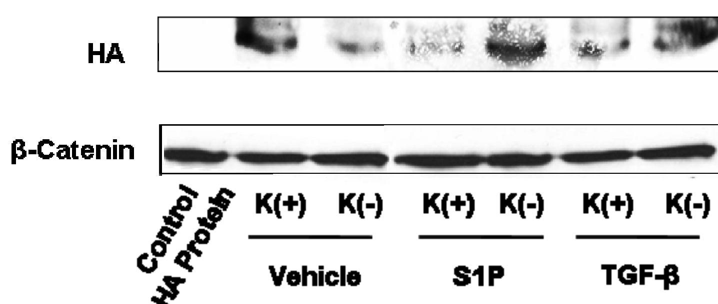


Fig. 35 Potassium depletion abrogates S1P-induced TβR-I internalization – Fibroblasts were firstly mock transfected (control HA protein) or with the HA-TβR-I plasmid (1 µg, 24 h) and then stimulated under conditions of potassium depletion (K(-)) or in the presence of isotonic conditions K(+)) as described in Materials and Methods. After stimulation with control vehicle (0.4% BSA/PBS), 1 ng/ml TGF-β, and 10^{-6} M S1P, the cold membrane fraction of the cells was electrophoresed and immunoblotted with an anti-HA-Probe antibody. The PVDF membrane was then stripped off and blotted against β-Catenin as control for membrane integrity.

3.3.9. Heterodimerization of $S1P_1$ and TβR-I

Having established that TβR desensitization with subsequent internalization occurred upon S1P stimulation and thus, TβR is intrinsically involved in S1P actions, the next step was to explore the potential physical association between TβR-I and $S1P_1$. Therefore, fibroblasts were at first transfected with the HA-TβR-I construct followed by stimulation with S1P and TGF-β. Immunoprecipitates of TβR-I-expressing fibroblasts with anti-HA antibodies and protein G-plus agarose were subsequently analysed by western blot analysis. As presented in Fig. 36 (lower panel), precipitated lysates of transfected fibroblasts showed equal amounts of basal HA-tagged TβR-I protein. Most interestingly, development with anti-$S1P_1$ antibodies revealed polypeptides of apparent molecular mass 42 kDa ($S1P_1$), consistent with the immunoprecipitation of the complex TβR-I and $S1P_1$ that were only partially separated by the electrophoresis conditions employed. Furthermore, the TβR-I-$S1P_1$ receptor complex was only formed when fibroblasts were stimulated with S1P (Fig. 36, upper panel). At the same time, no significant differences in band intensity were observed in cells incubated in the presence of TGF-β or vehicle.

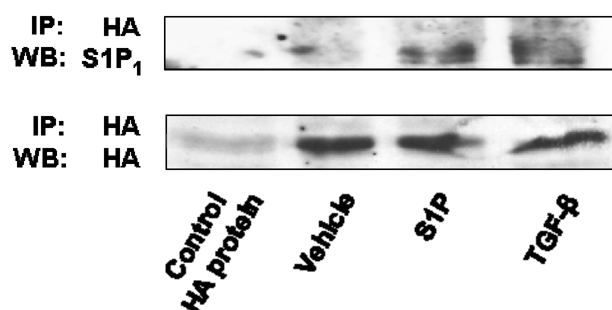


Fig. 36 Immunoprecipitation of both TβR-I and $S1P_1$ after S1P stimulation – Fibroblasts were firstly mock transfected (control HA protein) or with the HA-TβR-I plasmid (1 µg, 24 h) and then stimulated with control vehicle (0.4% BSA/PBS), 1 ng/ml TGF-β, and 10^{-6} M S1P. Lysates were afterwards immunoprecipitated with an anti-HA-Probe antibody and electrophoresed. Receptors complexes were detected by immunoblotting with an anti-$S1P_1$ antibody (upper panel) and the amount of transfected HA-TβR-I with anti-HA-Probe antibody (lower panel).

These experiments suggest a complex interplay between both S1P and TGF-β receptors to affect S1P-mediated Smad activation and chemotaxis.