4 DISCUSSION

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4.1. Role of S1P and TGF-β in the migration of human dermal fibroblasts

4.1.1. Receptor-induced chemotactic activity of S1P in human fibroblasts

The present study has demonstrated that the platelet-derived lysophospholipid mediator S1P exerted a signaling cascade activation involving Smad proteins and consequently motility via a receptor-mediated mechanism.

A number of chemokines, growth factors, cytokines, and other inflammatory mediators have been shown to stimulate directed cell migration, whereas a much more limited number of biological mediators have been described to modulate cell motility in a manner dependent on their concentration gradients (Yokomizo et al., 1997). Consistent with this concept, dermal fibroblasts showed this typical bell-shaped migration curve upon S1P stimulation. S1P exhibited a chemotactic effect in a concentration range from 10^{-6} to 10^{-9} M. 10^{-5} M resulted in a loss of migration, whereas 10^{-10} -10^{-12} M were effective evoking chemokinesis.

Although it is well established that S1P acts as a ligand for S1P receptors to regulate cytoskeletal changes and migratory responses, a few studies suggest that the effect of S1P on migration is likely mediated through intracellular actions rather than through cell surface receptors. However, the observation that PTX reduced chemotaxis towards S1P strongly argued against this possibility. The migratory response to exogenously added S1P was completely sensitive to PTX, indicating the involvement of Gi proteins and excluding the idea of an intracellular mechanism of action. Furthermore, the concentration of S1P in serum (about 484 nM), likely to be the highest in biological fluids (Yatomi et al., 1997), is above the EC_{50} levels of the observed receptor-mediated effects, but still below the concentrations required for the proposed intracellular effects (An et al., 2000). Finally, S1P can be released from platelets in response to other agonists such as thrombin, which makes S1P a likely candidate as an extracellular (patho)physiological mediator (Yatomi et al., 1995). S1P has been demonstrated to be unique as an extracellular regulator of motility as it exerts either stimulatory or inhibitory actions on cell motility (Takuwa, 2002). These bimodal actions are apparently cell type specific. Thus, S1P stimulates chemotaxis in

endothelial cells (Lee et al., 1999) (Okamoto et al., 2000b), keratinocytes (Sauer et al., 2004a), some glioma cells (Van Brocklyn et al., 2003), immature langerhans cells (Radeke et al., 2005), and at low concentrations, T cells (Graeler and Goetzl, 2002), whereas it inhibits cell migration in breast cancer cells (Wang et al., 1999) and melanoma and fibrosarcoma cells (Sadahira et al., 1992). In some cases, S1P enhances VSMC migration (Boguslawski et al., 2002), while inhibiting movement of these cells in others (Ryu et al., 2002). It has been shown that this bimodal regulation by S1P is based upon a diversity of S1P receptor isotypes (Okamoto et al., 2000a) (Ryu et al., 2002) (Takuwa, 2002).

Real-time PCR analysis demonstrated that the five known subtypes of S1PRs are expressed at significant levels in primary fibroblasts. A number of studies have described the $S1P_1$ and $S1P_3$ receptors as potent chemoattractants involved in the migratory response to S1P in a variety of cells (Rosenfeldt et al., 2001b; Taha et al., 2004; Takuwa, 2002). It is also well known that the $S1P_2$ either exhibit no effect on motility (Kon et al., 1999) or works as an antimigratory receptor by counteracting $S1P_1$ and $S1P_3$ (Takuwa, 2002) (Sugimoto et al., 2003) (Goparaju et al., 2005). Novel results presented $S1P_4$ as a receptor involved in motility through activation of the Rho GTPase Cdc42 (Kohno et al., 2003), while there are no evidences of a participation of $S1P_5$ in migration. The results of this work have implicated $S1P_1$ and $S1P_3$ as responsible for the S1P-induced migratory response in dermal cells, while $S1P_2$, $S1P_4$ and $S1P_5$ were ineffective in stimulating cell migration. Furthermore, these data confirmed the results obtained by Kon et al. in CHO cells expressing only $S1P_1$, $S1P_2$ and $S1P_3$, which give $S1P_1$ and $S1P_3$ a role in chemotaxis, but announced the $S1P_2$ as ineffective in inducing any effect in motility (Kon et al., 1999).

Many reports provide evidence that the activation of the Rho family small GTPase, Rac, Rho, and Cdc42, are involved in cytoskeletal rearrangement, as well as in cell migration. Thus, Rho mediates stress fiber formation and FA to negatively modulate migration (Sugimoto et al., 2003), while Rac and Cdc42 direct peripheral actin assembly that results in the formation of lamellipodia and filopodia, respectively, at the leading edge to promote motility (Hall, 1998) (Kohno et al., 2003). These work showed that S1P treatment led to increased actin polymerization at the leading edge and reorganisation of cytoskeleton, as well as promoting FA turnover, events necessary for a migratory response. Miura et al. indicated that S1P induces FAK phosphorylation could be abolished using the Rho inactivator C3 transferase, while

PTX partially inhibited FAK phosphorylation (Miura et al., 2000b). Ohmori et al. (Ohmori et al., 2001) provided evidence that PTX-sensitive migratory processes can be attributed to the receptor $S1P_1$ whereas PTX-insensitive, FAK-mediated migration results from $S1P_3$ receptor activation, as this receptor subtype also couples to Gq/G12/13 and could be blocked by the GPCR antagonist suramin.

The difference in the expression of the S1PR subtype might be responsible for the contradictory effects, stimulatory or inhibitory, on S1P-induced migration. $S1P_1$ and $S1P_3$ mediated S1P-directed, PTX-sensitive chemotaxis and Rac activation despite concurrent $S1P_3$-mediated stimulation of Rho via G12/13. Gi exerts a stimulatory regulation for Rac, which antagonizes and completely reverses G12/13-mediated inhibitory regulation of Rac (Sugimoto et al., 2003). These could indicate that integration of signals from Gi and G12/13 determines cellular Rac activity, which directs migration towards or away from a GPCR agonist. Since $S1P_2$, as well as $S1P_3$, is connected to Gi, but also to G12/13 and Gq, that lead to antagonistic responses, there must be a competitive process to stimulate Rac through Gi and promote actin accumulation and motility or to stimulate Rho (G12/13) that activate stress fibber formation and inhibition of motility. Thus, indicating a delicate balance of S1P effects dependent on the signal coupling of its receptor subtypes.

In conclusion, two ranges of active S1P-concentrations were provided within this work, 10^{-6} – 10^{-9} M evoked chemotaxis, whilst 10^{-10} – 10^{-12} M induced chemokinesis. Furthermore, $S1P_1$ and $S1P_3$ through Gi activation of the small G protein Rac might be involved in actin assembly for lamellipodia formation and consequentially PTX-sensitive chemotaxis of human dermal fibroblasts.

4.2.1. Migratory activity of TGF-β in human dermal fibroblasts

Although it is remarkable for an effector to both stimulate and inhibit motiltiy, it is not unprecedented. Indeed, the growth factor TGF-β1 induces analogues actions; for example, TGF-β mimics the action of S1P on migration and proliferation of dermal and epidermal cells (Vogler et al., 2003). Furthermore, TGF-β is in analogy to S1P a strong inhibitor of epidermal cells proliferation, but enhances proliferation of fibroblasts as well as activation of ECM production by fibroblasts (Sauer et al., 2004a). Moreover, it is a very potent chemoattractant and an inducer of its own production in monocytes (Ashcroft and Roberts, 2000). As well as S1P, TGF-β

showed to positively stimulate dermal fibroblasts motility although the active concentration 1 ng/ml was ineffective promoting chemotaxis, but rather induced chemokinesis. Migration of fibroblasts is crucial for reepithelialisation suggesting a role of TGF-β and S1P on cutaneous wound closure. Interestingly, the effects of TGF-β on fibroblast proliferation and its effect to induce chemotaxis are consistent with a positive role in wound healing, whereas the growth inhibitory effect on keratinocytes is not. Indeed, studies of wound healing in mice, in which the TGF-β gene has been deleted by homologous recombination, revealed that the release of the growth factor from degranulating platelets or secretion by infiltrating macrophages and fibroblasts is not critical to initiation or progression of tissue repair. Surprisingly, wounds showed an overall reduction in the amount of granulation tissue and an increased rate of epithelialisation compared with littermate controls suggesting that endogenous TGF-β may actually retard wound closure (Massague, 1999).

4.2.2. Participation of the Smad system and relevance of the MAPK pathway in the migration of fibroblasts

The present data revealed not only an activation of Smad2/3 after Smad phosphorylation and complexation with Smad4, but also the Smad nuclear accumulation in response to S1P. Moreover, Smad3 and ERK1/2 were involved in the chemotaxis and chemokinesis respectively. It has already been well characterised that both Smad3 and ERK1/2 are required for TGF-β migration in different cell types (Ashcroft et al., 1999) (Sauer et al., 2004b) (Radeke et al., 2005) (Malek et al., 2006) (Janda et al., 2006). Usually, C-terminal phosphorylation by the TβR-I is the key event in Smad2 and Smad3 activation (Massague and Chen, 2000). However, other kinase pathways further regulate Smad signaling as suggested by the complex phosphorylation patterns of endogenous Smads. It has been reported that also MAPK activation causes phosphorylation of Smads at specific sequences. ERK phosphorylates serine residues in the linker regions of Smad1 (Kretzschmar et al., 1997), Smad2, and Smad3 (Kretzschmar et al., 1999) and inhibits nuclear translocation of Smads and thus, signaling. Further, ERK1/2 activation by its upstream Ras GTPase leads to degradation and decrease in Smad4 expression and complex formation (Saha et al., 2001). Similarly, CamKII can phosphorylate Smad2

in vitro at linker-region residues Ser240 and Ser260 (as well as at Ser110 of the MH1 domain), which again inhibits nuclear translocation and signaling (Abdel-Wahab et al., 2002; Wicks et al., 2000). Significantly, phosphorylation of Ser240 was observed *in vivo* upon treatment of cells with EGF or PDGF (Matsuura et al., 2005) (Yoshida et al., 2005). PKC phosphorylates Smad2 *in vivo* and *in vitro* at Ser47 and Ser110, and Smad3 at the analogous Ser37 and Ser70 (Yakymovych et al., 2001). PKC phosphorylation of Smad3 blocks DNA-binding and consequently transcriptional regulation. All those phosphorylation sites are separate from the SSXS motif and lead to an inhibition of nuclear translocation of the protein probably due to a failed formation of the active complex R-Smad-Co-Smad. Nevertheless, the molecular mechanisms of synergistic effects on Smad2/3-mediated transcriptional responses by these molecules, which phosphorylate unknown residues outside the SSXS motif, also need further investigation. Since it was seen that S1P can stimulate ERK1/2 phosphorylation in fibroblasts, as well as in various cells types (Hsieh et al., 2006) (Donati et al., 2005) (Xin et al., 2004) (Cuvillier et al., 1996), it was important to show that S1P stimulation leaded to a functional Smad3-Smad4 complex. This was confirmed firstly, by the nuclear transport of Smad2/3 and then, by the detection of Smad4 in Smad1/2/3 immunoprecipitates after S1P stimulation. Addition of S1P resulted in an increased heteromerization, demonstrating that Smad3 is functionally activated by S1P. Further, detection of not-bonded cytoplasmatic Smad4 was diminished in stimulated cells, favoring the idea of complex formation, although questioning the loss in detectable Smad4. Smad4 is believed to shuttle continuously between the nucleus and cytoplasm with nuclear retention promoted by the formation of a heterotrimer with phosphorylated Smad2/3. Eventually, Smad2/3 becomes dephosphorylated and Smad4 returns to the cytoplasm (Pierreux et al., 2000), whereas Smad2/3 can be ubiquitinated and lost through proteasome-mediated degradation (Lo and Massague, 1999). In view of the decrease on Smad4, it remained unclear why TGF-β or S1P would cause a rapid loss in total detectable Smad4. One possibility might be that the epitope recognized by the Smad4 antibody is blocked by the oligomerization of Smad4 with Smad2 or Smad3, an interaction that occurs through the carboxyl-terminal domain of Smad4 (Wu et al., 1997). In this case, the apparent rapid disappearance of Smad4 following TGF-β or S1P stimulation of Smad phosphorylation would reflect and confirm the rapid formation of protein complex. The conclusive proof that Smad3 protein is not only activated, but

also plays a pivotal role in biological responses to S1P, is the loss of chemotaxis in Smad3-deficient fibroblasts. Murine cells stimulated with S1P failed to migrate in a concentration-dependent manner according to the chemotactic effect of S1P (10^{-6} - 10^{-9} M). Not only dermal but also epidermal cells were able to activate Smad complexes after S1P stimulation. Sauer et al. already described a requirement of Smad3 in effects of S1P on the modulation of keratinocytes migration and proliferation (Sauer et al., 2004a). All these data point out the Smad system as a mediator of S1P signaling. However, evidences were given for the participation of MAPK in the fibroblasts chemokinesis induced by S1P, as the enhanced migration after KO cells stimulation with low concentrations of S1P (10^{-10} – 10^{-12} M) resulted in a loss of migration when interfering with the MAPK cascade.

The data presented in this work demonstrated that $S1P_1$ and $S1P_3$ were required for both the Smad activation and the PTX-sensitive migratory response to S1P. At the same time, pre-incubation with PTX interfered with the ability of S1P to activate R-Smads, indicating that the activation event and motility were consequences of S1P receptor stimulation and moreover confirmed the specific role of S1P on Smad3 activation. In addition, the fact that Smad activation occurs after 15 min S1P-stimulation clearly argued the possibility that secreted TGF-β was the responsible for the Smad activation. In fact, our group measured whether treatment of human fibroblasts with S1P leads to a secretion of the cytokine TGF-β. Indeed, S1P neither enhanced peptide levels of TGF-β nor increased latent complexes of TGF-β over a time period of 24 h. To further substantiate that S1P-induced fibroblasts effects are independent of TGF-β release, α-smooth muscle actin expression was measured under immunoneutralized conditions on a fluorescence microscope. As already published, S1P effects on fibroblasts were done in the presence of anti-TGF-β antibodies (Keller et al., 2007).

4.2. Crosstalk between S1P and TGF-β signaling

4.2.1. Relevance of TβRs on the actions of S1P

TGF-β can synergistically act with different transduction pathways to either enhance a response or evoke a new one. Concomitant signaling through RTKs in many developmental and biological systems suggests that certain intermediates in their signaling pathways might be shared. TGF-β was originally identified for its ability to transform normal rat kidney fibroblasts *in vitro*, an effect that was dependent on the presence of EGF (Roberts et al., 1983). TGF-β and EGF synergistically stimulate epithelial to mesenchymal transition (EMT) through a common MEK-dependent mechanism (Grande et al., 2002). Similarly, TGF-β can synergistically act with FGF on proliferation partially through PDGF (Bosse et al., 2006). Recent progress has revealed that Smad signaling is not merely determined by activation of the class of TGF-β receptors, but is also regulated through crosstalk with other kinase signaling cascades. In addition, the Smads regulate transcription through functional cooperativity and physical interactions with other transcription factors, which might also be targets for regulation by other signaling cascades (Zhang and Derynck, 1999). Recently, it was shown that either HGF or EGF stimulate phosphorylation of endogenous Smad2 that mediate activating signals from RTKs (de Caestecker et al., 1998). HGF antagonizes TGF-β signaling by stabilizing the Smad transcriptional corepressor TGIF (TGF-β-interacting factor), thereby sequestering TGF-β-Smad-mediated gene transcription (Wen et al., 2005). Inhibition of Smad1 signaling by RTKs (Kretzschmar et al., 1997) suggests that Smad proteins may play a pivotal role in mediating crosstalk between serine/threonine kinase receptors and RTKs. In addition, Smad proteins provide an interface between signals from TGF-β and the steroid hormones. Thus, the liganded glucocorticoid receptor interacts with the MH2 domain of Smad3 to repress its transcriptional activating activity (Song et al., 1999). In contrast, Smad3 interacts with the vitamin D receptor through its MH1 domain, acting as an inducible coactivator to potentiate the activity of the vitamin D receptor on its response element in a ligand-dependent manner (Yanagisawa et al., 1999). According to this, the idea that S1P could share with TGF-β the Smad transduction system seemed to be viable. Fanayan et al. described that the

insulin-like growth factor binding protein-3 (IGFBP-3) could stimulate Smad2/3 phosphorylation, potentiate TGF-β-stimulated Smad phosphorylation, and cooperate with exogenous TGF-β in cell growth inhibition (Fanayan et al., 2000). Fanayan et al. has not yet elucidated whether IGFBP-3 act as a ligand for TβRs or cross-activate the TGF-β pathway, but elicited the requirement of functional TβRs to evoke this effects (Fanayan et al., 2002). Although no evidences of synergism were observed, the fact that functional active TβRs were involved highlighted the significance of active TβRs in the effects of IGFBP-3. In a similar extend, this work provided evidences that S1P requires the presence of functional TβRs for the Smad-dependent effects on human dermal fibroblasts. Cells treated with SB431542 an inhibitor of the TβR-I kinase, and therefore without functional TβRs, failed to both migrate and activate the Smad system towards S1P.

4.2.2. S1P GPCR trans-activation

During the last years, it has become evident that different types of receptors including GPCRs not only undergo homodimerization, but also heterodimerization or oligomerization with other receptor classes and thereby trigger a crosstalk between different signaling cascades (Devi, 2001). This mechanism of signal modulation would be very useful to understand how S1P possesses contradictory effects especially regarding motility. Whereas $S1P_3$ stimulates migration through Rac activation, its homolog $S1P_2$ inhibits motility by activating a different member of the Rho GTPases family, RhoA. G protein counteracting after heterodimerization of two S1PRs could explain why the balance of promoting or inhibiting migration is shifted to one or the other direction. In this context, it is worth mentioning that S1PRs not only cross-activate other S1PRs or GPCRs (Carrillo et al., 2003), but also other growth factor signaling cascades. A number of studies have indicated an important role of some receptors in the GPCR signaling (Endo et al., 2002) (Kim et al., 2000) (Le Stunff et al., 2004) (Tanimoto et al., 2002). A transactivation between VEGFR, EGFR (Sukocheva et al., 2006) (Kim et al., 2000), PDGFR (Goparaju et al., 2005) and S1PR, which leads to a regulation of the mitogenic response and motility in various cell types was already described (Endo et al., 2002; Tanimoto et al., 2002) (Donati et al., 2005). In particular, it has been well established that the PDGFR not only transactivates $S1P_1$ (Rosenfeldt et al., 2001a) (Rosenfeldt et al., 2001b) (Long et al.,

2006), but also $S1P_2$ signaling (Donati et al., 2005) (Goparaju et al., 2005). Waters et al. have recently shown that SB649146, a novel inverse agonist of the $S1P_1$ receptor, reduced the endocytosis of the PDGF-β receptor-$S1P_1$ receptor complex as well as the stimulation of ERK1/2 MAPK and cell migration in response to PDGF in murine embryonic fibroblasts (Waters et al., 2006). Recently, it has also been mentioned a crosstalk between TGF-β and S1P signaling in keratinocytes (Sauer et al., 2004a), inmatures langerhans cells (Radeke et al., 2005), and renal messangial cells (Xin et al., 2004).

The tendency of GPCRs to interact with other signaling cascades together with the fact that S1P-mediated Smad activation was PTX sensitive and required active TβRs presume a cross-activation between S1P and TGF-β receptors. Indeed, the interaction of both receptors was observed in fibroblasts transfected with the HA-tagged TβR-I after 30 min S1P stimulation. The $S1P_1$ and TβR-I were co-immunoprecipitated from cell lysates using an anti-HA antibody and detected with an antibody against the $S1P_1$. These findings suggest that these receptors form a complex in fibroblasts. The important feature of the complex is that the close proximity association between the TβR-I and the $S1P_1$ receptor permits the use of activated G protein subunits (made available by the constitutively active $S1P_1$ receptor) by the TβR to modulate signal transmission. In case of fibroblasts, this adjacency of S1PRs negatively affected the TGF-β response.

4.2.3. Heterologous desensitization of TGF-β signaling by S1P

Receptor desensitization represents an important physiological "feedback" mechanism that protects against both acute and chronic receptor overstimulation. Receptor desensitization also acts to filter information from multiple receptor inputs into an integrated and meaningful biological signal through inactivation of weaker receptor-mediated signals. However, receptor desensitization can also significantly limit the therapeutic usefulness of many receptor agonists. This mechanism of regulation has been characterized for GPCR signaling including $S1P_1$ in i.e. RBL-2H3 cells (Jolly et al., 2004). This effect could also be confirmed in fibroblast's S1PRs. Short-term exposure of the agonist profoundly internalized $S1P_1$ GPCR subtype in cells transfected with the GFP-tagged $S1P_1$, as a result cell migration was abolished. TGF-β pre-incubation of fibroblasts also showed a homologous desensitization of the

TGF-β migratory activity towards agonist stimulation due to surface receptor down-regulation. There have been conflicting reports whether TβRs undergo ligand-mediated internalization. Earlier studies have been contradictory and ranged from no significant down-regulation to a 50% decrease in surface binding (Frolik et al., 1984) (Wakefield et al., 1987). However, those initial studies suggesting that a large intracellular pool of recycling receptors replenished cell surface binding after internalization, were based upon the results of ligand binding to both endogenous heteromeric and homomeric TβRs and/or the earlier consideration that the type III receptor (beta glycan) was the primary signaling receptor for TGF-β. More recent studies (Zhao and Buick, 1995) (Muramatsu et al., 1997) (Anders et al., 1997) show that TβR-I and TβR-II are down regulated and that distinct endocytic effects are observed for TβR heteromers and homomers. Homomeric receptor interactions are signaling incompetent (Anders and Leof, 1996) (Luo and Lodish, 1996) (Muramatsu et al., 1997) thus, only signaling-competent heteromeric receptors would be down regulated. In general, studies on the role of internalization on TGF-β-activated receptor systems have supported the concept that activated TβRs internalize and remain localized into endosomes for a substantial amount of time (Hayes et al., 2002). The general idea consists now that like many other cell surface receptors, TβRs are internalized upon ligand stimulation (Lu et al., 2002b). Once ligand binding occurs, typically a growth factor receptor is removed from the plasma membrane by an endocytic process, resulting in receptor down-regulation. According to this, it was expected that sensitized fibroblasts respond to TGF-β in a less extend. Interestingly, S1P also exhibited the ability to down regulate the TβR from the cell surface, as well as interfering with the TGF-β-mediated Smad2 phosphorylation and chemotaxis. Pre-treatment of cells with S1P for 30 min before stimulation induced an internalization of S1P receptors possibly together with the TβRs to activate the Smad system, as TGF-β-mediated actions were manifestly diminished in desensitized cells. It has been argued that S1P may act as an endogenous inhibitor of TGF-β signaling, since overexpression of SphK1, a crucial enzyme in the formation of S1P, interferes with induction of the COL1A2 promoter by TGF-β (Sato et al., 2003). In contrast to an endogenous role of S1P, evidences are provided, at least in fibroblasts, that S1P influences TGF-β signaling through activation of GPCR, since signaling is completely abolished in the presence of PTX. Neither serum nor pre-incubation of fibroblasts with TGF-β showed any influence on the chemotaxis induced by S1P, indicating a

specific role of TGF-β in the mechanism of action of the lysophospholipid. The issue goes beyond this and points out a crosstalk between a serine/threonine kinase receptor and a GPCR.

4.2.3.1. Role of ERK1/2 MAPK in the counteracting effects of S1P on TGF-β signaling

S1P effects are mediated by various pathways, which are part of an extremely complex signalling network. Further experiments were performed with the objective to specify, which of the pathways are causally involved in the inhibition of TGF-β mediated chemotaxis of S1P sensitised fibroblasts. The feasibility that the effects of S1P were carried out by the MAPK system has already been discused. Therefore, the possibility that the inhibitory effects of S1P on TGF-β signaling were ruled out through ERK1/2-induced Smad inactivation were further analysed. Firstly, an active complex of R-Smad and Smad4 were detected in the nucleus in stimulated cells. Afterwards and regarding migration, ERK1/2 MAPK appeared to be involved in the Smad-independent chemokinesis induced by low concentration of S1P. Nevertheless, inhibition of ERK1/2 activation did not show any influence on the inhibitory effects of S1P on TGF-β signaling. If the mechanism of action of S1P on fibroblasts chemotaxis were independent of the TβR-I, but would involve the MAPK pathway that cross-activates the Smad system, interfering with the MAPK cascade would have reversed the inhibition of motility. On the contrary, incubation with the PD098059 was unable to reverse the S1P-inhibitory regulation of TGF-β response in fibroblasts despite MAPK blockade, indicating a specific action of S1P on TGF-β actions.

4.2.4. TβR-I clathrin-mediated endocytosis induced by S1P

Receptor endocytosis has long been regarded as an attenuation mechanism to switch off receptor signaling. However, accumulating evidences suggest that endocytosis may facilitate signaling by targeting signaling complexes to specific subcellular localization, either to increase access of activated receptor kinases to their substrates or to compartmentalize signaling complexes (McPherson et al., 2001) (Di Fiore and De Camilli, 2001). Endocytosis consists of several interconnected pathways, including the initial internalization of receptors, sorting

endosomes, recycling of receptors back to the plasma membrane, and/or shunting to proteosomes/lysosomes for degradation. The activated TβR complex undergoes endocytosis via two distinct routes, for two different purposes: it is internalized via coated vesicles to early endosomes for signaling and via caveolae to caveolin-positive vesicles for degradation (Di Guglielmo et al., 2003). Thus, segregation of TβRs into distinct endocytic compartments regulates Smad activation and receptor turnover (Di Guglielmo et al., 2003). Interactions between SARA and Smad2/3 allow more efficient recruitment of R-Smads to the receptors for phosphorylation in early endosomes (Tsukazaki et al., 1998) (Wu et al., 2000) (Hayes et al., 2002). To facilitate this process, activated receptor complex is internalized via clathrin-coated pits (Lu et al., 2002b). New coated pits initially appeared as planar clathrin lattices that increased in size by the formation of polygons at the margin of the lattice. Once the lattice reached a critical size, it invaginated to form coated vesicles. This fact indicated that the clathrin lattice of coated pits is actively involved in membrane shape change during endocytosis and that the structural proteins of the lattice are cyclically assembled and disassembled in the process. Larkin et al concluded that potassium (K+) depletion inhibited the assembly of coated pits, but that existing coated pits in K+-depleted cells were able to internalize (Larkin et al., 1986). The finding that TβR-I is internalized upon S1P stimulation together with the colocalisation of both $S1P_1$ and TβR-I and that there was essentially no information concerning the mechanism(s) through which TβRs were internalized, made the question whether TβR-I internalize via clathrin-coated pits interesting to determine.

Correlating with the migratory results, TGF-β induced receptor internalization, as TβR-I disappeared from the cell surface. Furthermore, transfection of the HA-TβR-I followed by stimulation with S1P evoked a reduction of TβR-I from the surface indicating an interaction of both receptors to promote signaling. Both internalization processes were disrupted by potassium depletion suggesting an effect mediated by clathrin-coated pits. These results were in agreement with earlier studies that used granulocyte macrophage colony-stimulating factor-TGF-β chimeric receptors in which the internalization of these chimeric receptors was abolished by potassium depletion (Anders et al., 1997). Rapid disruption of clathrin-mediated endocytosis through potassium depletion is an acute procedure, thus decreasing the probability of inducing compensatory responses that could occur with other inhibitory methods.

The fact that clathrin-mediated receptor pinocytosis cease in the endosome suggests that in addition to its role in establishing correct traffic patterns of internalized proteins, the endosome might form an essential part of the signal transduction machinery of the cell. The endosome may provide a specialized environment, analogous to those established within the plasma membrane by the localized enrichment of specific lipids (Sedwick and Altman, 2002). This hypothesis was well analysed by Hayes et al. resulting in the observation that TβR-clathrin vesicles fusion with the endosome to translocate R-Smad in the nucleus and thereby extending the role of the endosome to that of a compartment specialized for the propagation of certain extracellular signals (Hayes et al., 2002).

4.3. Conclusion

In conclusion, the present work distinguished between two ranges of active S1P concentrations. S1P at low doses induced an ERK MAPK-mediated chemokinesis in a Smad-independent manner, whereas higher concentrations exhibited a chemotactic effect. Hence, it was identified a signaling pathway through which S1P chemotaxis was mediated by the activation of cell surface receptors $S1P_1$ and $S1P_3$ and through the participation of Gi proteins. A pathway that required the presence of TβR-I and the Smad3 protein. Furthermore, the S1PR-TβR-I complex was internalized via clathrin-mediated endocytosis to transduce signaling.

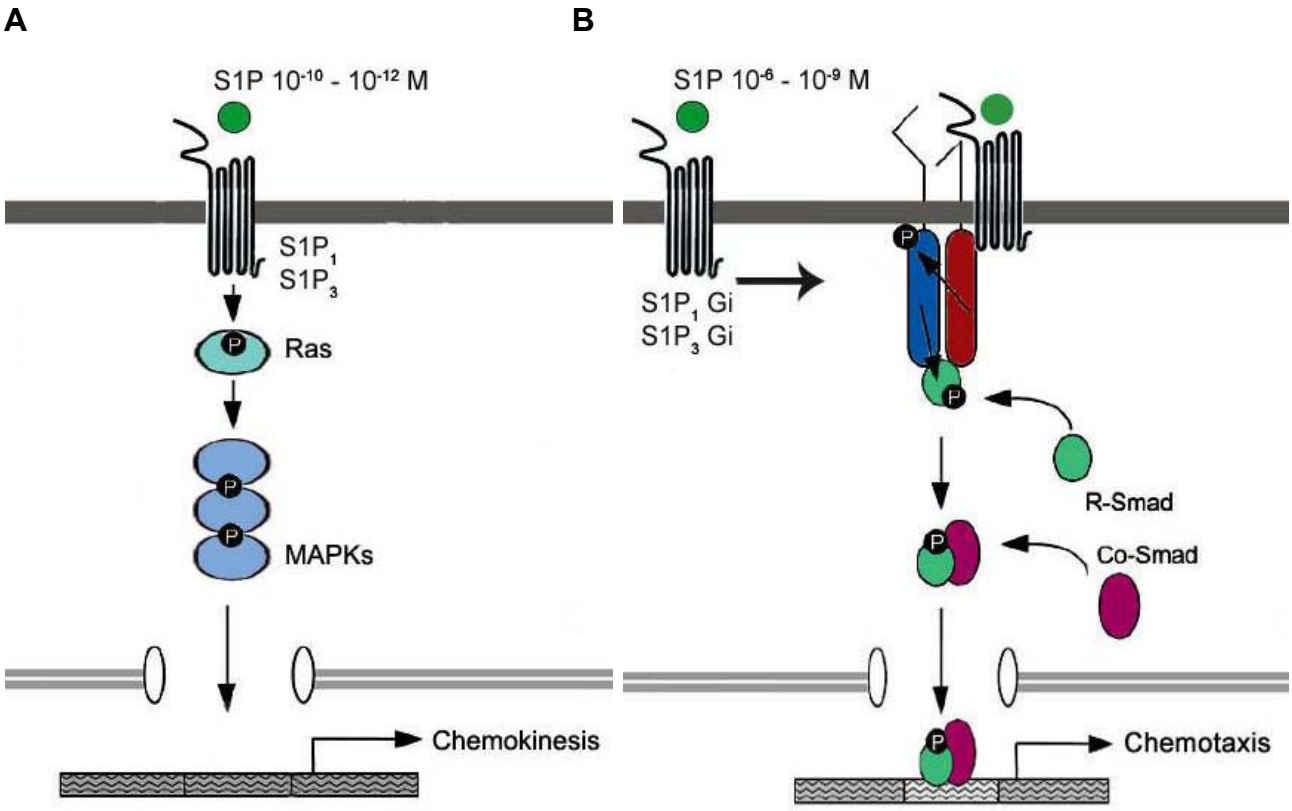

Fig. 37 Schematic representation of conclusions.

4.4. Outlook

The present work provides an insight into the signaling pathway of the lysophospholipid S1P, which is currently still not well characterised. New upstream molecules like S1P are appearing to affect the Smad system through receptor activation. Further research objectives could include which promoter or repressor reporter genes are involved in the activation of the TGF-β cascade by S1P. Therefore, it has to be documented which combination S1P receptor subtype, Smad proteins, and transcription factors are responsible for other responses mediated by S1P. Studies of heterodimeric S1PR pairs would support a mechanism in which agonists at GPCRs mediate transactivation of the GPCR-associated G proteins. Furthermore, heterocomplexes between serine/threonine kinase receptors, especially other members the TGF-β family, and G proteins coupled to S1P receptors remained in outlook. A series of issues have been raised about the meaning and validity of receptor dimerization data that rely exclusively on co-immunoprecipitation. Therefore, the receptor-receptor interaction that occurs after physical proximity remained to be monitored by new techniques of energy transfer after protein-protein interaction. The preceding data support the hypothesis that the primary mechanism for TβR internalization is clathrin-dependent. Furthermore, S1P induced punctuate localisation of the FYVE-protein SARA with Smad around the nucleus and this distribution was difused and thorough the cytosol when cells were treaten with wortmanin, an inhibitor of the PI3K activity. These results are not presented in this work but give more evidences for a role of clathrin-coated pits and endosomes. However, the use of specific clathrin inhibitors would be necessary in order to confirm clathrin-dependent endocytosis. Since TβRs can internalize via clathrin-coated pits or caveolin vesicles, the significance of these lipids-rafts in signaling remained to be elucidated.

Developing selective tools for the resolution of function across this broad lipid receptor family and other signaling as the serine/threonine kinase TβR families would help developing new therapeutical resource. This increasing field of application can be extended to many pathophysiological conditions such as the dysregulation of wound healing processes that lead to disrupted scar formation and fibrotic tissue, hyperproliferative skin disorders, fibrosis, and tumors and metastasis. Agonists such

as FTY720, SEW2871 or KRP-203 or inverse agonist, SB649146, and antagonists like suramin or JTE-013 are now being used. Understanding the molecular pathways involved in their signaling would be very helpful to develop new therapies and avoid undesirable adverse reactions as happened to the immunosuppresor FTY720. FTY720 that binds to $S1P_{1, 3, 4, 5}$, is currently in phase III clinical trials as monotherapy for relapsing remitting multiple sclerosis. In a previous phase III trial of kidney transplantation, FTY720 was found to be effective, albeit with some adverse effects including bradycardia due to signaling associated to $S1P_3$ (Sanna et al., 2004). The novel immunomodulator, KRP-203, which binds unique to $S1P_1$ and has structural similarity to FTY720, induces lymphocytopenia and shows considerable immunosuppressive effects on heart and skin allograft. Interestingly, and in contrast to FTY720, KRP-203 has a lower potential to induce heart rate reduction in guinea pigs (Fujishiro et al., 2006). Understanding the contribution of individual receptors has been limited by the embryonic lethality of the $S1P_1$ KOs and the unavailability of selective agonists or antagonists. The general approach of identifying receptor-selective pharmacological tools should allow the contributions of other S1P receptors to be clarified, and the hierarchy and signaling mechanisms of receptor usage in different physiological systems will become clear and potentially therapeutically useful over time.