# 5 SUMMARY

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Connective tissue is a highly vascularized tissue that forms the supporting and connecting structure of the body. It represents an essential system for all vertebrate organisms, as it emerges as indispensable for cell shape, protection, and motility. The regulation of connective tissue homeostasis by cytokines and growth factors plays a decisive role in several biological processes such as embryo-, angio-, and tumorgenesis, fibrosis or wound healing. The extracellular matrix (ECM) represents the portion of tissue secreted by connective tissue cells, predominantly fibroblasts, in the intercellular space. The interaction between ECM and connective tissue cells results in the induction of adhesion, migration, proliferation of cells as well as in the assembly, disassembly and remodeling of the tissue. Therefore, the regulation of fibroblast motility in the ECM is a crucial aspect to maintain the homeostasis of different types of connective tissue. Mainly, the motility mechanism involves a cycle of four steps: protrusion of the leading edge, adhesion to the substratum, retraction of the rear, and de-adhesion. These effects are basically conducted by the complex interplay of leading edge formation and focal adhesions turnover.

The lysophospholipid sphingosine 1-phosphate (S1P) is released from degranulating platelets at the ECM and acts as an intracellular and extracellular messenger that regulates a broad spectrum of biological aspects, among others motility. Previous studies demonstrated that S1P regulates both positively and negatively the migration of different cell types. S1P is known as a ligand of five heterotrimeric G protein-coupled receptors ($S1P_{1-5}$). Therefore, the presence of different receptor subtypes was analyzed. A real-time PCR analysis unveiled for the first time the presence of mRNA transcripts of all five known S1P receptors ($S1P_3$ >> $S1P_1$ > $S1P_2$ > $S1P_4$, $S1P_5$) in human dermal primary fibroblasts. In this work, the effects of S1P on dermal cell motility and their underlying molecular mechanisms were explored. This data presented that extracellular added S1P induced a migratory activity on fibroblasts in a concentration dependent manner via its receptors. S1P exhibited a chemotactic effect in a concentration range from $10^{-6}$ to $10^{-9}$ M, whereas $10^{-5}$ M resulted in a loss of migration and $10^{-10}$ - $10^{-12}$ M were effective evoking chemokinesis. Moreover, inhibition of Gi signaling after pertussis toxin treatment clearly reduced motility towards S1P. This fact did not only confirm the contribution of S1P receptors, but also suggested an involvement of subtype-specific Gi proteins. To

further elucidate S1p receptors are involved in the migration process, antisense experiments were performed. The results manifested that $S1P_1$ and $S1P_3$ were responsible for the S1P-induced migration. Correlating with the significantly increased fibroblasts migration, S1P caused both extension of lamellipodia at the cell periphery of human fibroblasts with a rearrangement of the cytoskeleton and focal adhesions turnover. These effects were revealed by phalloidin staining of actin filaments and by staining of the adhesion protein vinculin. The data presented here correspond to events necessary for a migratory response. The chemotactic response of fibroblasts towards S1P was comparable to the migratory behavior induced by low concentrations of the transforming growth factor β (TGF-β). TGF-β is analogously to S1P, secreted by activated platelets and is found in the damaged ECM at high concentrations i.e. wound areas of the dermis. Both mediators exert analogous bimodal effects on migratory and proliferative responses of epidermal cells. Unlike S1P, the signaling pathways of TGF-β are well characterized. The major signal cascade involves ligand binding to TGF-β type I (TβR-I)- and type II (TβR-II)-receptors followed by R-Smad (Smad2/3) phosphorylation and heteromerization with the co-Smad (Smad4). In this active form, the Smad complex translocates to the nucleus and regulates gene activity. In this context, the ability of S1P to activate the Smad system was researched. Indeed, S1P activates Smad2/3 in a concentration and time dependent manner with a similar kinetic as TGF-β. Furthermore, S1P induced a Smad2/3 nuclear accumulation in a same extension as TGF-β did. In addition, the results revealed that $S1P_1$ and $S1P_3$ receptors and their Gi proteins contribute to the Smad activation, as well as to the cell migration. Previous reports on Smad3 knockout (KO) wounds have shown that Smad3 is responsible for the migratory effect of TGF-β on keratinocytes and monocytes. According to this, the role of this signaling protein in the biological responses of fibroblasts towards S1P and TGF-β should be investigated. The present outcomes indicated that analogously to TGF-β, the S1P-induced chemotaxis of Smad3 KO fibroblasts was almost totally diminished. However, disruption of the Smad system did not show any effect on the chemokinesis upon S1P. Since it has often been described that ERK-MAPK participates in the regulation of motility in different cell types, the idea of an involvement of this kinases in the chemokinetic effect of S1P was keenly presumable. Indeed, results on fibroblasts showed an ERK1/2 phosphorylation upon S1P and pointed at a role of the MAPK system in its cell migration, as treatment with

the MEK inhibitor, PD098059, clearly interfered with the chemokinesis induced by S1P.

Due to the similarities found on the S1P and TGF-β effects on fibroblasts and the results of previous experiments on keratinocytes, which suggested the involvement of TβRs in the cellular effects of S1P, the role of these receptors in fibroblast actions was further analyzed. Results revealed inhibitory effects on S1P-caused migration and Smad2/3 activation when fibroblasts were treated with the TβR-I inhibitor, SB431542 and confirmed in that way the involvement of TβR-I. An unexpected result occurred when cells were firstly pre-incubated with S1P and then analyzed according to their migratory behavior in response to TGF-β. Under these circumstances, desensitized fibroblasts were not able to migrate and TGF-β failed to induce a Smad2-phosphorylation. Since pre-treatment of fibroblasts with S1P already abrogates the TGF-β-evoked Smad2-phosphorylation and Smad proteins are directly phosphorylated by activated TβRs, an interaction of TGF-β- and S1P-receptors was conceivable. This kind of crosstalk has already been published for $S1P_1$ and different tyrosin-kinase receptors like the PDGFR. Therefore, cells were transfected with a HA-epitope tagged TβR-I and then checked for functionality by investigation of migration to TGF-β. Western blot analysis of the cold membrane fraction of transient transfected cells revealed a complete internalization of TβR-I in response to S1P, as well as to TGF-β. Furthermore, acute abrogation of clathrin-dependent endocytosis through potassium depletion caused no change in the amount of membrane TβR-I in response to both, S1P and TGF-β. To further substantiate the concept of a crosstalk between both signaling pathways, an immunoprecipitation of the TβR-I followed by western blot analysis for $S1P_1$ was performed. The results showed the dimerization of $S1P_1$ and TβR-I after stimulation of fibroblasts with S1P, supporting in that way the idea of a direct interaction of these receptors.

In conclusion, the present work distinguishes 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 is mediated by the activation of cell surface receptors $S1P_1$ and $S1P_3$ and Gi proteins. This pathway required the presence of the Smad3 protein, which is activated and translocated in nucleus by S1P and TβR-I, which is internalized via clathrin-mediated endocytosis.