1 INTRODUCTION

1. INTRODUCTION

1.1. Lysophospholipids

Lysophospholipids are a family of biological lipid mediators. Phospholipids and their metabolites were originally known as components of the cellular lipid bilayer, but only recently, their role as intercellular signaling molecules has been appreciated. Two of the best-studied lysophospholipids, Sphingosine 1-phosphate (S1P) and Lysophosphatidic acid (LPA), signal through cognate G protein-coupled receptors (GPCRs) to activate many well-known intracellular signaling pathways, leading to a variety of biologically important cell responses. Lysophospholipids and their receptors were found in a wide range of tissues and cell types, indicating their importance in many physiological processes, including reproduction, vascular development, embryogenesis, and nervous system function.

1.1.1. S1P origin

Sphingolipids are a family of lipids mediators, which general structure is composed of a polar head group and two nonpolar tails. The core of sphingolipids is the long-chain amino alcohol, sphingosine (Sph Amino acylation with a long chain fatty acid at carbon 2 of sphingosine yields a ceramide (N-acylsphingosine) (Cer) whereas phosphorylation at carbon 1 leads to S1P (Fig. 1).

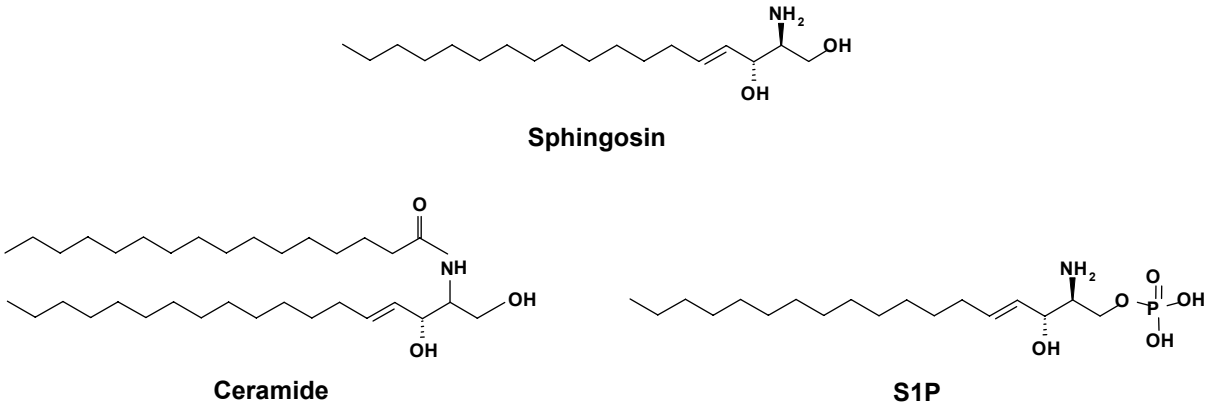

Fig. 1 Structures of the principal sphingolipids

The knowledge about the sphingolipid metabolism is gaining in complexity. The sphingomyelin (SM) pathway is a signaling system that has been conserved from yeast to humans. The generation of Cer, the central molecule in this pathway, may involve hydrolysis of SM by various sphingomyelinases (SMases) or *de novo* synthesis by condensation of L-serine and palmitoyl-CoA. Once generated, Cer may transiently accumulate or be converted into various metabolites. Phosphorylation by ceramide kinase generates ceramide 1-phosphate (Cer1P), while deacylation by various ceramidases yields sphingosine, which may then be phosphorylated to S1P (Fig. 2).

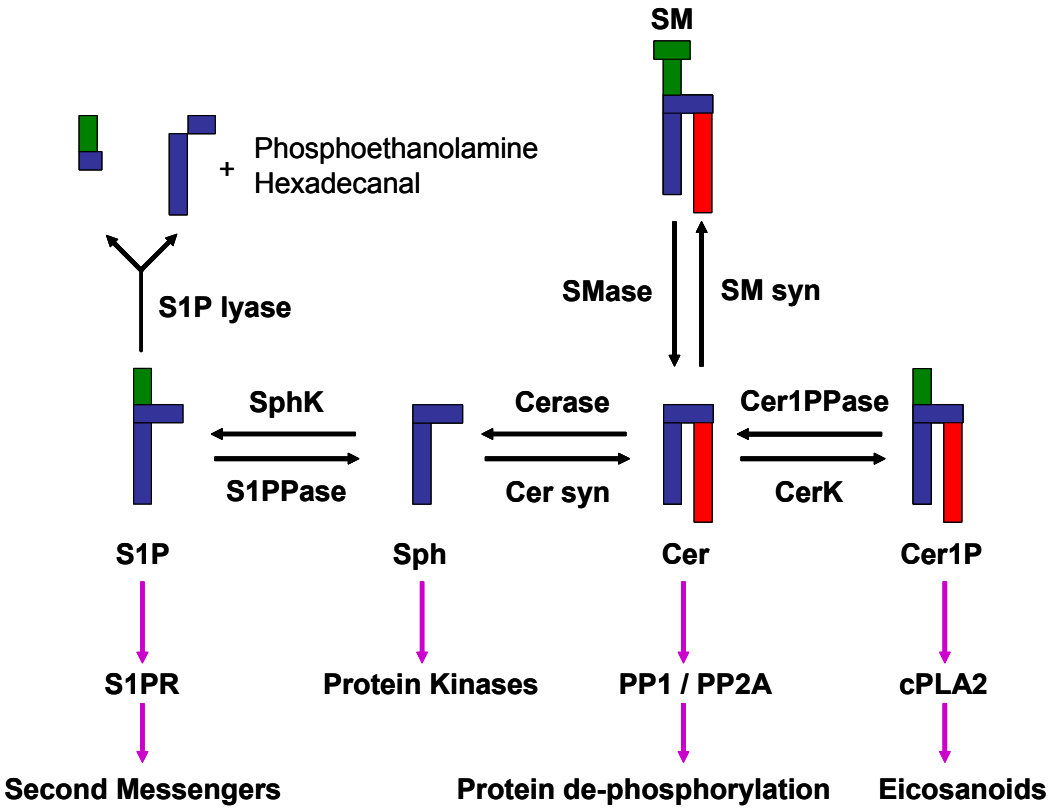

Fig. 2 *Illustration of pathways involved in the sphingolipid metabolism of eukaryotic cells. The panel shows the structure of some sphingolipids. Note that, for simplicity, the phosphate group (green) and only one kind of sphingoid base (sphingosine, in blue) are shown to which only one kind of fatty acid (palmitic acid, in red) is* N*-acylated. SM, sphingomyelin; SM syn, SM synthase; SMase, sphingomyelinase; Cer, ceramide; Cer syn, Cer synthase; Cerase, ceramidase; Cer1P, ceramide 1-phosphate; Cer1PPase, Cer1P phosphatase; CerK, Cer kinase; Sph, sphingosine; S1P, sphingosine 1-phosphate; SphK, Sph kinase; S1PPase, S1P phosphatase; S1PR, S1P receptors; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; cPLA2, cytosolic phospholipase A2.*

The intracellular concentration of S1P greatly varies within cell types. The balance between its synthesis, catalyzed by sphingosine kinase (SphK) and its metabolism tightly regulates the S1P level. S1P degradation is catalyzed by irreversible cleavage through endoplasmic reticulum S1P lyase and reversible dephosphorylation to sphingosine by specific S1PPases, and still not well-characterized enzymes with phosphohydrolase activities (Spiegel and Kolesnick, 2002). Two distinct SphK isoforms, SphK1 and SphK2, have been cloned and characterized (Kohama et al., 1998) (Liu et al., 2000a). Diverse external stimuli, particularly growth and survival factors, stimulate their activation. S1P is secreted into the bloodstream by platelets upon activation (Yatomi et al., 1995) (Tani et al., 2005) and by mast cells (Olivera and Rivera, 2005), as well as by a variety of other cell types.

In the blood, S1P is present at high concentrations (Yatomi et al., 1997) roughly 0.1-1 μM. Nevertheless, many of the physiologically relevant functions occur in the low nanomolar range, including chemotaxis (Lee et al., 2006b), activation of endothelial nitric oxide synthase (eNOS) (Igarashi and Michel, 2001), and inhibition of lymphocyte recirculation (Sanna et al., 2004). Free plasma levels of S1P are tightly regulated by protein binding to albumin and high-density lipoprotein (Murata et al., 2000) to avoid deleterious effects of systemic S1P receptor subtype activation at high concentrations of ligand.

Although sphingolipids were originally thought to predominantly play a structural role as components of the lipid bilayer (Kolesnick, 1987), sphingolipid metabolism is now known to be a dynamic process. Furthermore, sphingolipid metabolites, including Cer, Sph, and S1P, are active mediators that play essential roles in cell growth, survival, and death. Recent studies have placed these sphingolipids in the centre of a number of important biological processes, specifically in signal transduction pathways. S1P like its precursors Sph (Anliker and Chun, 2004) and Cer (Futerman and Hannun, 2004) are important for calcium homeostasis (Meyer zu Heringdorf et al., 1998), cell growth (Olivera et al., 1999) (Sauer et al., 2004a), and apoptosis (Maceyka et al., 2002). Olivera et al. showed that intracellular S1P formed by overexpression of SphK1 promoted growth and survival (Olivera et al., 2003) whereas SphK2 and its product, S1P, may contribute to apoptosis-inducing properties (Maceyka et al., 2005). Moreover, the dynamic balance between levels of the sphingolipid metabolites, Cer and S1P, and consequent regulation of opposing

signaling pathways is an important factor that determines whether a cell survives or dies, thereby putting the fate of the cell in a life and death balance.

1.1.2. S1P receptors (S1PRs)

GPCRs are seven transmembrane proteins that form the largest single family of integral membrane receptors. Classical models predicted that GPCRs function as monomers, but several recent studies acknowledge that GPCRs exist as dimeric or oligomeric complexes (George et al., 2002) (Bai, 2004). GPCRs transduce information provided by extracellular stimuli into intracellular second messengers via coupling to one or multiple heterotrimeric guanine nucleotide binding proteins (G proteins) and the subsequent regulation of a variety of effector systems(Rodbell, 1997).

At present, ten distinct lysophospholipid receptors have been identified in mammals therefrom five for LPA, another bloodborne lipid that is structurally similar to S1P and has several overlapping cellular effects, and five for S1P. S1PR have similar KD values in the nanomolar range (Watterson et al., 2003). Each of the five S1PRs has significant homology displaying 40-50% amino acid identity and approximately 60% amino acid similarity (Zhang et al., 1999) (Im et al., 2000) (Yamazaki et al., 2000). The $S1P_1$ receptor subtype has been the first family member to be cloned and has been identified as an immediate early transcript in phorbol ester-induced endothelial cell differentiation (Hla and Maciag, 1990), leading to the former designation endothelial differentiation gene (EDG)-1. According to the international union of pharmacology (IUPHAR), the currently accepted naming system is $S1P_{1–5}$ (Chun et al., 2002): $S1P_1$ (EDG1), $S1P_2$ (EDG5), $S1P_3$ (EDG3), $S1P_4$ (EDG6), and $S1P_5$ (EDG8). Several other orphan receptors have been implicated as potential S1PRs, including GPR3, 6, 12, and 63, although these remain unconfirmed and little is known regarding the significance of these receptors (Kostenis, 2004).

1.1.2.1. Agonist-induced desensitization

The exposure of GPCRs to agonists often results in a rapid attenuation of receptor responsiveness. This process, termed desensitization, has traditionally been defined as the process whereby a GPCR-initiated response plateaus and then diminishes despite the sustained presence of agonist. Desensitization is the consequence of a

combination of different mechanisms. These mechanisms include the uncoupling of G protein in response to phosphorylation by both second messenger-dependent protein kinases and G protein-coupled receptor kinases (GRKs) (Ferguson, 2001), and subsequently binding of β-arrestin molecules (Lefkowitz and Whalen, 2004). Next occurs the internalization of cell surface receptors to intracellular membranous compartments (Anborgh et al., 2000) via clathrin-coated pits or lipid-raft endocytosis, where trafficking to acidic early endosomes leads to recycling of the resensitized GPCR back to the cell surface, or targets the receptor for degradation (Ferguson, 2001). Finally, the down-regulation of the total cellular complements of receptor due to reduced mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors takes place (Luttrell, 2006). Nevertheless, several recent studies have suggested that the molecular processes that desensitize signaling pathways at the plasma membrane can simultaneously initiate alternate pathways after receptor clustering and internalization (Lefkowitz, 1998). GPCRs share this common regulatory pathway that governs agonist-induced desensitization and endocytosis, which is necessary not only for signal termination, but also for receptor degradation and recycling, as well as for certain signaling events (Lefkowitz and Whalen, 2004).

1.1.2.2. Physiological relevance of S1PRs

S1PRs are characterized for ubiquitary tissue localization. $S1P_{1-3}$ have wide tissue distribution; $S1P_4$ is mainly found in lymphoid tissues; and $S1P_5$ is predominately expressed in the nervous system (Jaillard et al., 2005). Studies on S1PR knockout (KO) mice show the importance of their expression for normal development. $S1P_1$ gene deletion was lethal to embryos because of defective vessel maturation (Liu et al., 2000b). Moreover, while $S1P_2$- and $S1P_3$- null mice were viable, fertile, and normally developed (MacLennan et al., 2001) (Ishii et al., 2001), deletion of both $S1P_2$ and $S1P_3$ was responsible for marked perinatal lethality (Ishii et al., 2002). $S1P_4$, was cloned from *in vitro* differentiated murine and human dendritic cells and show potential immune modulatory functions (Graler et al., 1998), however $S1P_4$ KO mice have not yet been reported. Homozygous $S1P_5$ deficient mice have no apparent behavioral deficit, and neuropathological examination of $S1P_5$ null brain do not show

any evident myelin deficiency; although, $S1P_5$ deficient cells can differentially influence specific stages of oligodendrocyte development (Jaillard et al., 2005).

1.1.3. G protein signaling

S1P is a pleiotropic molecule that exerts diverse activities on various cell types. S1P was known as lipid mediator that acts as a second messenger (Spiegel and Merrill, 1996). Although now there is no doubt that S1P acts extracellularly, several studies still support the notion that this potent lipid also have intracellular functions. S1P can act autocrine and paracrine; thus, has novel dual actions signaling inside and outside the cell.

S1PRs productively couple to three different types of Gα chains: Gi, Gq and G12/13, and β/γ dimers. Most of these receptors are capable of differentially coupling to one or multiple G proteins and intracellular effectors in that way initiating a multiple of distinct signal cascades. Guanosine 5-[gamma-35S] triphosphate-gammaS ([35S]GTPγS) binding studies show that $S1P_1$ couples exclusively to Gi proteins (Windh et al., 1999) and, thus, responses mediated through this receptor are sensitive to pertussis toxin (PTX), which ADP-ribosylates and inactivates Gi proteins. Both, $S1P_2$ and $S1P_3$, couple principally to Gi, Gq, and G13 however, it appears that they do so with different preferences, leading to different biological responses (Windh et al., 1999). Whereas $S1P_4$ couples to both Gi and G12/13, but not Gq (Graler et al., 2003), $S1P_5$ just couples to Gi and G12 (Malek et al., 2001).

Interactions of all five receptors with Gi, which account for the decreases in intracellular cAMP (Anliker and Chun, 2004), mediate the stimulation of protein kinases that recruit the ras-raf cascade leading to activation of mitogen-activated protein kinases (MAPKs) as well as the activation of phospho-inositol 3 kinase (PI3K) (Tanimoto et al., 2002) that lead to Akt activation and eNOS phosphorylation (Morales-Ruiz et al., 2001). PI3K and Akt activation are associated to both chemotaxis (Lee et al., 2006b) and suppression of apoptosis induced by S1P (Limaye et al., 2005) (Fieber et al., 2006), while extracellular signal-regulated kinase (ERK) and PI3K activation are the most common ways involved in the mitogenic effects of S1P (Yamada et al., 2004) (Sorensen et al., 2003). On the contrary, the antiproliferative effects of S1P (Vogler et al., 2003) (Kim et al., 2004) (Yamashita et al., 2005) may involve ERK phosphorylation with transient Akt dephosphorylation

(Yamashita et al., 2005). $S1P_2$ and $S1P_3$ through their coupling to Gq/11, initiate phospholipase C (PLC) activation, which liberates inositol triphosphate (IP3) to mobilize intracellular Ca^{2+} and is capable of activating MAPKs directly or in some types of cells through intermediate induction by diacylglycerol (DAG) of protein kinase C (PKC) activity (Song et al., 2006). Interaction of $S1P_{2-5}$ with G12/13 initiates the Rho GTP pathways that contribute to serum response element (SRE)-mediated transcription as well as activation of phospholipase D (PLD) and PI3K (Dutt et al., 2004). G12/13 induction of Rho GTP-coupled activities is the sole mechanism for eliciting cytoskeleton-dependent effector responses (Meacci et al., 2003). Regarding motility, the members of the small GTPase Rho family, primarily Rac, Cdc42, and Rho, are well-known regulators of actin organization and myosin motor function. Rho mediates stress fiber formation and focal adhesion and therefore, seems to be involved in inhibition of motility in many cell types (Goparaju et al., 2005; Lepley et al., 2005). On the contrary, Rac and Cdc42 direct peripheral actin assembly that results in formation of lamellipodia and filopodia, respectively, at the leading edge. PI3K activation of Rac mediates S1P-induced motility through $S1P_1$ and $S1P_3$ via Gi (Okamoto et al., 2000a), while neither of these S1P receptors affect Cdc42 activity, the third member of the family (Sugimoto et al., 2003). Only $S1P_4$ activates Cdc42 leading to positive modulation of motility (Kohno et al., 2003). $S1P_5$ causes a decrease of ERK phosphorylation in a PTX insensitive manner although activates another member of the MAPK, the c-Jun N-terminal kinase (JNK) (Malek et al., 2001), which is involved in apoptosis.

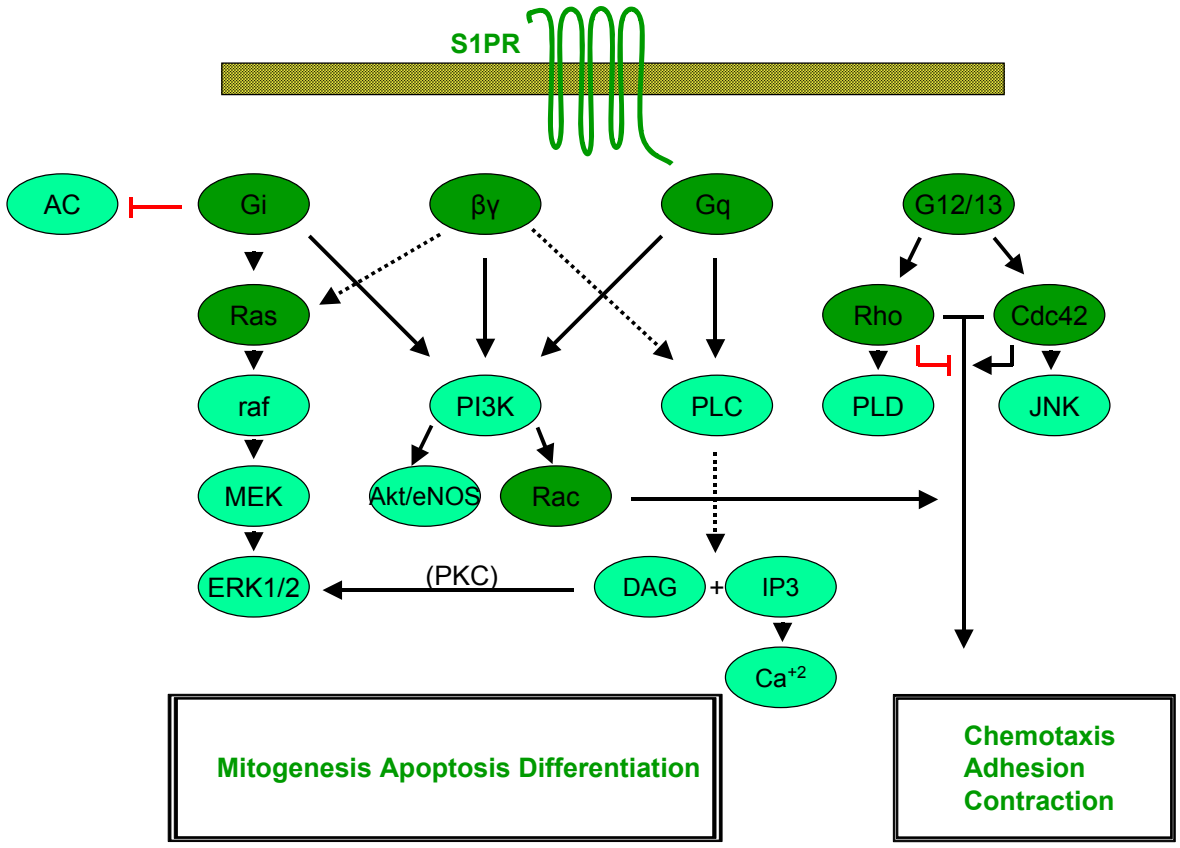

Fig. 3 *S1P signaling through G protein-coupled receptors. Coupling of S1P receptors with different classes of G proteins, activation or inhibition of downstream second messenger molecules, and the most prominent resultant cellular effects are illustrated. AC, adenylate cyclase; PI3K, phosphoinositol 3-kinase; PLC and D, phospholipase C and D; JNK, c-Jun N-terminal kinase; eNOS, endothelial nitric oxide sinthase; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; Ca^{+2}, calcium.*

The Gβ/γ dimers may participate directly by recruiting PLC and Rho GTP (Goetzl and An, 1998). The Gβ/γ subunits are also involved in the activation of a Src family tyrosine kinase followed by the subsequent tyrosine phosphorylation of the same downstream adaptor proteins used by tyrosine kinase receptors (RTKs) and seems to involve transactivation of these receptors (Pierce et al., 2000).

S1P has been shown to induce bimodal responses depending on cell type, receptor expression and G protein activation. S1P stimulates proliferation of several cell types (Van Brocklyn et al., 1998) (Huang et al., 1999) (Olivera et al., 2003), although it can inhibit proliferation in some other cases (Sauer et al., 2004a) (Kim et al., 2004) (Yamashita et al., 2005). S1P affects cell migration, again both positively and negatively (Okamoto et al., 2000a) (Kohno et al., 2003) (Goparaju et al., 2005).

Recapitulating, the cellular effects of S1P may be divided into two categories (Fig. 3). One cluster of growth-related S1P activities includes stimulation of proliferation, prolongation of survival, prevention and suppression of apoptosis, and alterations in differentiation. A second group of cellular effects of S1P is directed to functions dependent on the cytoskeleton such as shape changes, aggregation, adhesion, chemotaxis, contraction, and secretion. This second group of responses may be evoked by lower concentrations of S1P than the growth-related cluster or may require a concentration gradient. Some complex cellular responses may reflect participation of both types of primary responses. For example, the effects on cellular and tissue differentiation are attributable to both changes in expression of selected genetic programs, which share signaling pathways with other growth-related effects in the first cluster, and up-regulation of surface adhesive proteins that is related by signaling pathways more closely to the cytoskeleton-dependent group of effects in the second cluster.

1.1.4. GPCR crosstalk with other growth factors receptors

Numerous crosstalks can occur considering the activation and signal transduction of GPCRs. More than a single type of G protein is activated in physiological conditions upon activation of one kind of receptor and implies complex crosstalks among downstream effectors molecules. Finding the evidence for stimulation of one type of GPCR leading to the activation of other receptor-mediated signaling events has been the subject of numerous recent studies. Receptor dimerization, homo- and heterodimerization, implicated in the activation as well as signaling of GPCRs, might also give rise to synergistic or more complicated effects. Until very recently, the conventional assumption for GPCRs was that monomeric receptors interact allosterically with a single heterotrimeric G protein. However, recent evidence suggests that receptor dimerization or oligomerization is required for signal transduction by several GPCRs in a fashion similar to the non-GPCR receptor families such as RTKs and the growth hormone receptor family (Bouvier, 2001) (Milligan, 2001). There is increasing evidence for homo- and hetero-oligomerization of GPCRs (Hall et al., 1999), which suggests that dimerization/oligomerization may be important in different aspects including biogenesis, activation, and function of receptors. Hetero-oligomerization or crosstalk between different subtypes of GPCRs

may explain some aspects of the crosstalk regulation observed between different signaling systems. Bradykinin, a vasodepressor, is a functional antagonist of angiotensin II. In addition to the interconnection between these two hormone systems by the angiotensin converting enzyme (ACE), which releases angiotensin II from its precursor and inactivates bradykinin, it was suggested that this functional antagonism might also be achieved from mutual regulatory influences at the receptor level (Hur and Kim, 2002). Various studies have revealed that cellular responses to S1P and other GPCR agonists depend on the function of receptors with intrinsic tyrosine kinase activity in several cell systems (Prenzel et al., 1999) (Tanimoto et al., 2002), a phenomenon that was termed interreceptor crosstalk or RTK signal transactivation. Transactivation of GPCRs and RTK has been well defined as a mechanism of action for S1P (Rosenfeldt et al., 2001a) (Sukocheva et al., 2006). Pioneer studies demonstrated that GPCRs are able to transduce some proliferative signals through the activation of RTKs. There is a large amount of evidence indicating that proteolytic activities releasing growth factors trapped in the surrounding extracellular matrix (ECM) precedes GPCR-induced transactivation of RTKs (Selbie and Hill, 1998). For example, in primary rat smooth muscle cells the insulin-like growth factor-1 receptor (IGF-1R) phosphorylation is induced by thrombin (Weiss et al., 1997), while the vascular endothelial growth factor receptor type 2 (VEGFR-2) is transactivated by S1P in human umbilical vein endothelial cells (HUVECs) (Endo et al., 2002). Moreover, it was reported that LPA induces platelet-derived growth factor receptor (PDGFR) tyrosine phosphorylation in a cultured line of mouse fibroblasts (L cells) (Herrlich et al., 1998), and that opioid receptor agonists transactivate the fibroblast growth factor receptor (FGFR)-1 in rat C6 glioma cells (Belcheva et al., 2002) suggesting that transactivation of distinct RTKs can contribute to GPCR signaling in a cell-type-specific manner. It should be mentioned that recently published experimental findings demonstrated that the crosstalk between RTKs and GPCRs could also occur in the opposite direction. A novel mechanism, relevant to angiogenic events, indicated that PDGF-induced cell migration requires the presence of $S1P_1$ to activate Rac-dependent pathways (Hobson et al., 2001). Moreover, both S1P and PDGF stimulation of HEK293 cells induced $S1P_1$ - PDGF-receptor complex and ERK1/2 activation (Waters et al., 2006). Thus, deletion of $S1P_1$ suppressed Rac activation and the chemotaxis of fibroblasts (Hobson et al., 2001). However, not only RTKs can interact with GPCRs, the cross communication with

serine/threonine kinase receptors, such as TGF-β receptors, was already described in epidermal cells. Migration of keratinocytes towards S1P was markedly reduced when the TGF-β type I-receptor was blocked (Sauer et al., 2004a).

1.2. Transforming growth factor-β (TGF-β)

1.2.1. The TGF-β superfamily

The TGF-β family comprises a large number of structurally related polypeptide growth factors, each capable of regulating a fascinating array of cellular processes including cell proliferation, differentiation, motility, adhesion, and death. Expressed in complex temporal and tissue-specific patterns, TGF-β and related factors play a prominent role in the development, homeostasis, and repair of virtually all tissues in organisms, from fruitfly to human. Collectively, these factors account for a substantial portion of the intercellular signals governing cell fate (Massague, 1998). TGF-β is the prototype of the TGF-β superfamily, which in addition to the TGF-β isoforms 1-5 comprises activin, inhibins, Müllerian inhibitory substance, growth differentiation factors, and bone morphogenetic proteins (BMPs). The expression levels and receptor affinity of the TGF-β isoforms differ tissue-dependent and the effects appear to be slightly variable (Centrella et al., 1994). In mammalian organisms, there are three different TGF-β isoforms 1, 2, and 3, which function through serine/threonine kinase receptors. Apparently, TGF-β synthesis and secretion seems to be dependent on cell type and the kind of TGF-β isoform involved. The first member of the TGF-β superfamily of secreted polypeptide factors, TGF-β 1, was discovered approximately 25 years ago (Roberts et al., 1981) (Anzano et al., 1983). Since then, the family has grown considerably and now comprises over 30 vertebrate members and approximately a dozen structurally and functionally related proteins in invertebrates such as worms and flies. The bioactive forms of TGF-β and related factors are dimers held together by hydrophobic interactions. The dimeric structure of these ligands suggests that they function by bringing together pairs of receptors, forming heterotetrameric receptor complexes.

TGF-β is secreted from cells in a latent form. It requires activation before it binds to its cognate receptors and exerts its effects. Proteolytic processing separates an N-terminal propeptide from TGF-β. After processing, TGF-β noncovalently associates with its propeptide. Because this interaction prevents TGF-β from binding its receptors, the propeptide is termed latency-associated peptide (LAP) (Lu et al., 2002a). Within the secretory pathway, the complex of TGF-β and LAP often interacts

covalently with a fibrillin-like LTBP (latent TGF-β binding protein) to form large latent complex (LLC), which targets the TGF-β complex to the ECM (Oklu and Hesketh, 2000) (Roberts and Derynck, 2001). Before it can exert its biological activity, mature TGF-β must be released from this complex, as, for example, through proteolytic cleavage of the prosegment (Koli et al., 2005) or through some drugs, which are able to initiate pathways leading to the release of mature TGF-β (Minguillon et al., 2005).

1.2.2. TGF-β receptors (TβRs)

Based on their structural and functional properties, the TβR family is divided into two subfamilies: type I receptors (TβR-I) and type II receptors (TβR-II) (Fig. 4). Type I receptors have a higher level of sequence similarity than type II receptors, particularly in the kinase domain. The membrane-anchored proteoglycan betaglycan, also known as the TGF-β type III receptor, is a class of molecule that controls ligand access to receptors. TβR-III has long been known to mediate TGF-β binding to the type II receptor, thus acting as accessory receptor or coreceptor. This role is particularly critical for TGF-β2 (Shi and Massague, 2003).

Vertebrate type I receptors form three groups, whose members have similar kinase domains and signaling activities. In mammals, one group includes TβR-I, activin receptor-IB (ActR-IB), and activin receptor-like kinase 7 (ALK7), another includes bone morphogenetic protein receptor-IA (BMPR-IA) and -IB, and a different one includes ALK1 and ActR-I (Massague, 1998). Most type I receptors have received different names, since different investigators have simultaneously cloned them. One practice has been to use the neutral nomenclature ALK and to adopt a more descriptive name when the physiological ligand becomes known. Thus, the TGF-β type I receptor originally known as ALK5 (Franzen et al., 1993) is now called TβR-I (Yamashita et al., 1994). In vertebrates, the type II receptor subfamily includes TβR-II, BMPR-II, ActR-II and -IIB, and anti-müllerian hormone receptor (AMHR), which selectively bind TGF-β (Lin et al., 1992), BMPs (Liu et al., 1995), activin, and müllerian-inhibiting substance (MIS) (di Clemente et al., 1994), respectively.

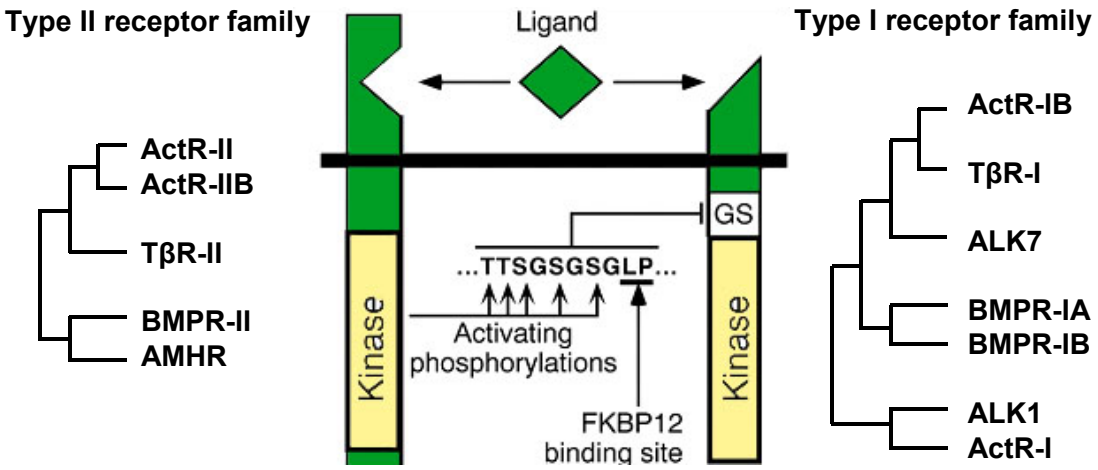

Fig. 4 *Type I and II TGF-β receptor families. In type I receptors, the protein kinase domain is preceded by the GS domain (GS). The characteristic GS sequence motif of TβR-I is shown, indicating the phosphorylation sites and the FKBP12-binding site. Listed members are from vertebrates. The dendrograms indicate the relative level of amino acid sequence similarity in the kinase domain.*

Receptors type I and II are glycoproteins of approximately 55 kDa and 70 kDa, respectively, with core polypeptides of 500 to 570 amino acids including the signal sequence (Attisano et al., 1992) (ten Dijke et al., 1993). The extracellular region is relatively short (approximately 150 amino acids), N-glycosylated (Wells et al., 1997), and contains 10 or more cysteines that may determine the general fold of this region. The transmembrane region and the cytoplasmic juxtamembrane region of type I and II receptors have no singular structural features. However, Ser213 in this region of TβR-II is phosphorylated by the receptor kinase in a ligand-independent manner and is required for signaling activity (Luo and Lodish, 1997). Ser165 in the juxtamembrane region of TβR-I is phosphorylated by TβR-II in a ligand-dependent manner, and this appears to selectively modulate the intensity of different TGF-β responses (Souchelnytskyi et al., 1996).

A unique feature of type I receptors is a highly conserved 30-amino acid region immediately preceding the protein kinase domain (Fig. 4). This region is called the GS domain because of the containing characteristic SGSGSG sequence. Ligand-induced phosphorylation of the serines and threonines in a TTSGSGSG sequence of TβR-I by the type II receptor is required for activation of signaling (Wrana et al., 1994). The GS domain is a key regulatory region that controls the catalytic activity of

the type I receptor kinase and its interaction with substrates (Wieser et al., 1995). Immediately following the GS domain, all type I receptors have a Leupeptine-Proline (LP) motif that serves as a binding site for the immunophilin FKBP12 (Chen et al., 1997). FKBP12 may act as a negative regulator of the receptor signaling function. (Huse et al., 2001). The kinase domain in type I and II receptors correspond to the canonical sequence of a serine/threonine protein kinase domain (Franzen et al., 1993). Consistent with this, type I receptors have been shown to phosphorylate their substrates, whereas type II receptors phosphorylate themselves and type I receptors on serine and threonine residues (Souchelnytskyi et al., 1996).

The most commonly accepted receptor model for TGF-β actions is a heteromeric receptor complex at the cell surface composed of two type II and two type I transmembrane receptors. This model involves direct binding of TGF-β to the type II receptor and subsequent interaction of this complex with the type I receptor, which, in effect, becomes recruited into the complex. Type I receptors can recognize ligand that is bound to the type II receptors but not ligand that is free in solution (Attisano et al., 1993).

1.2.3. Smad proteins and the Smad signal transduction pathway

The proteins of the Smad family are the first identified substrates of type I receptor kinases. They are intracellular signal transducers that play a central role in the transduction of receptor signals to target genes in the nucleus. The founding member of the Smad family is the product of the *Drosophila* gene Mad (mothers against dpp) and the *Caenorhabditis elegans* gene Sma (Sekelsky et al., 1995). The human genome encodes eight Smad family members (Moustakas et al., 2001). Functionally, Smads fall into three subfamilies: receptor-activated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5, Smad8, which become phosphorylated by the type I receptors; the common mediator Smad (Co-Smad): Smad4, which oligomerizes with phosphorylated R-Smads; and inhibitory Smads (I-Smads): Smad6 and Smad7, which are induced by TGF-β family members. The latter exerts a negative feedback effect by competing with R-Smads for receptor interaction and by marking the receptors for degradation. Substrate specificity is determined by the L45 loop in the type I receptors and, primarily, by the L3 loop in the R-Smad C-terminal domain;

thus, TGF-β and activin receptors phosphorylate Smad2 and Smad3, and BMP receptors phosphorylate Smad1, Smad5 and Smad8 (Chen et al., 1998).

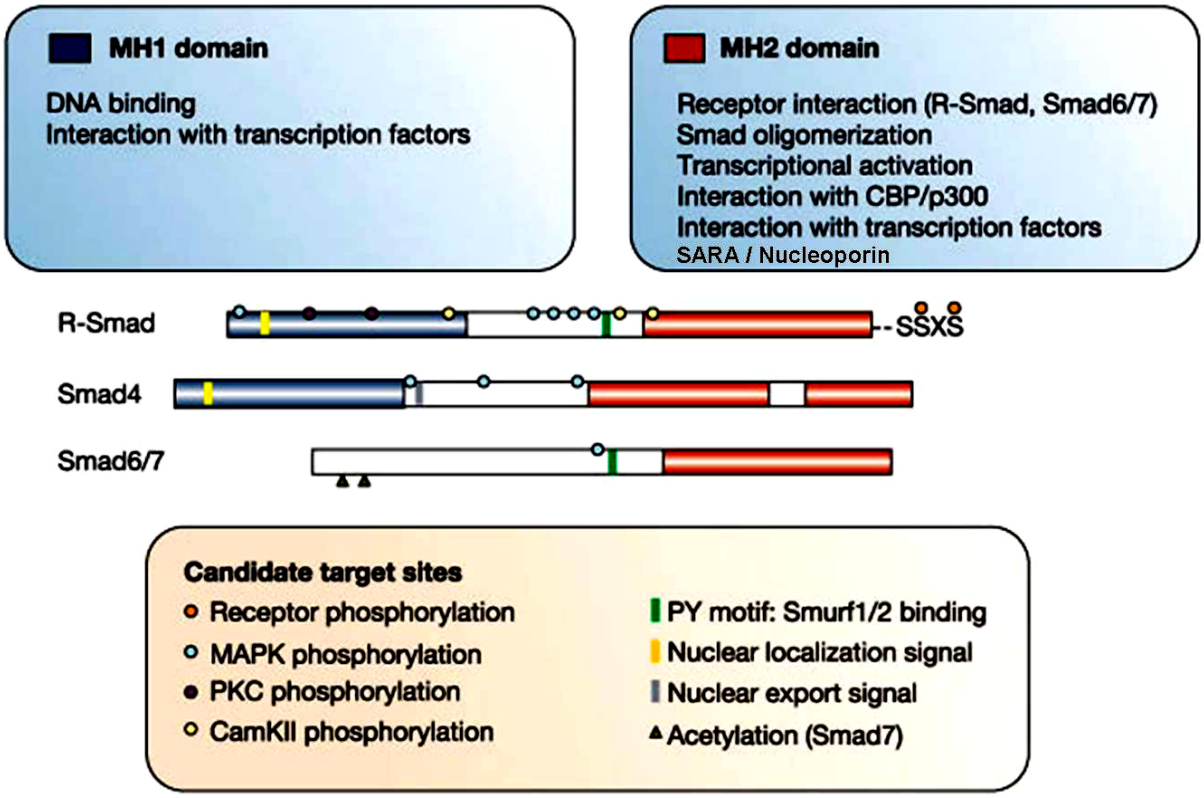

Fig. 5 *Structural organization and role of the domains of Smads, and candidate target sites for kinase pathways. Such pathways include ERK MAPK and JNK, as well as CamKII and PKC. The significance of candidate MAPK phosphorylation sites in Smad4 and Smad6/Smad7 is not known.*

Smads have two conserved domains, the N-terminal Mad homology 1 (MH1) and C-terminal Mad homology 2 (MH2) domains (Fig. 5). The MH1 domain is highly conserved among R-Smads and Co-Smads; however, the N-terminal parts of I-Smads have only weak sequence similarity to MH1 domains. The MH1 domain regulates nuclear import and transcription by binding to DNA and interacting with nuclear proteins. The MH2 domain is also highly conserved among all Smads. Its structure contains several α-helices and loops, which surround a β-sandwich (Shi, 2001), and it resembles the forkhead-associated (FHA) domain, a phosphopeptide-binding domain common in transcription and signaling factors (Li et al., 2000). The

MH2 domain regulates Smad oligomerization, recognition by type I receptors and interacts with cytoplasmic adaptors and several transcription factors.

The recruitment and phosphorylation of R-Smads by the TβR complex is regulated by several proteins, which have anchoring, scaffolding and/or chaperone activity (Tsukazaki et al., 1998) (Miura et al., 2000a). Among them, the Smad anchor for receptor activation (SARA) regulates the subcellular distribution of Smad2 and Smad3, and presents the R-Smads for phosphorylation to the activated TβR complex (Tsukazaki et al., 1998). The regulation of the localization of SARA occurs through its FYVE (Fab1p, YOTB, Vac1p, EEA1) domain zinc finger. The corresponding domain present in EEA1 and SARA binds phosphatidylinositol 3-phosphate (PtdIns(3)P) and has been implicated in the localization of these proteins to the early endosomal compartment (Itoh et al., 2002a). The FYVE domain functions to direct SARA to the membrane through interactions with PtIns(3)P and is required to maintain the normal localization of SARA, but is not involved in mediating interaction with Smads (Tsukazaki et al., 1998). The interaction between SARA and Smad2 or Smad3 occurs via a Smad-binding domain (SBD) in SARA and the MH2 domain in Smad2; this interaction appears to be highly specific since SARA does not interact with either Smad1 or Smad5, which mediates the BMP signaling pathways and shares greater than 80% sequence similarity with Smad2. SARA competes with the nucleoporins, involved in nuclear import of Smad2/3 and Smad4, for the binding sites (Xu et al., 2003b). The SBD in turn functions to bind unactivated R-Smads, thus recruiting the receptor substrate to this subcellular region. Once localized to this region, the C-terminal domain of SARA functions with R-Smad bound to the SBD to promote interaction with the receptor complex. Formation of a ternary complex involving Smad2/3, SARA, and TβR-I was postulated to facilitate efficient phosphorylation of Smad (Tsukazaki et al., 1998) (Runyan et al., 2005).

Once Smads are presented to receptors, phosphorylation occurs. Phosphorylation of the C-terminal serine residues in R-Smads by type I receptor kinases is a crucial step in TGF-β family signaling (Abdollah et al., 1997) (Macias-Silva et al., 1996) (Roberts et al., 2001). The two outmost C-terminal serine residues become phosphorylated and, together with a third, non-phosphorylated serine residue, form an evolutionarily conserved SSXS motif in all R-Smads (Souchelnytskyi et al., 1997). Unphosphorylated Smad proteins exist primarily as monomers and upon phosphorylation, R-Smads form homo-oligomers, which quickly convert to

hetero-oligomers containing Smad4 (Correia et al., 2001) (Kawabata et al., 1998). The obligatory consequence of R-Smad phosphorylation is the formation of oligomeric complexes with the Co-Smad, Smad4. Shi et al showed that oligomeric Smads might exist as a trimer with the Co-Smad (Shi and Massague, 2003). Biochemical and structural evidence suggests that the phosphorylated C-terminal tail of R-Smads interacts specifically with the L3 loop of another Smad, which is sufficient to cause their oligomerization (Correia et al., 2001).

The activated R-Smads in complex with the Co-Smad is then translocated into the nucleus, where it regulates transcription of ligand-responsive genes (ten Dijke et al., 2000). Through a conserved β-hairpin loop in the MH1 domaim Smad3 and Smad4 bind directly but with low affinity to Smad-binding elements (SBEs), which have the minimal sequence motif 5'-CAGAC-3'. In contrast, Smad2-Smad4 can not bind to the SBE because of its unique exon-3-encoded sequence (Moustakas et al., 2001). Nuclear import and activation of transcription occurs by a cytosolic-factor-independent import activity that requires a region of the MH2 domain (Xu et al., 2000). Posttranslational modifications such as phosphorylation and ubiquitination are a common regulatory mechanism associated with TGF-β signaling. Ubiquitin ligases, designated Smurf (Smad ubiquitination regulatory factor), ubiquitylate the Smad complex and target it for proteosomal degradation (Moustakas et al., 2001).

1.2.3.1. Receptor-mediated endocytosis

It has been shown that ligand-bound TβRs endocytose to early endosomes, where they phosphorylate Smads. However, whether TGF-β can signal without receptor internalization is still in question (Zhou et al., 2004). In the absence of ligand, TβR endocytose constitutively and come recycled back to the plasma membrane (Ehrlich et al., 2001) (Di Guglielmo et al., 2003). TβR-II contain an internalization signal belonging to the dileucine family (Ehrlich et al., 2001), which is one of the motifs recognized by the clathrin-associated adapter complex AP2 (adaptor protein 2) (Traub, 2003). In addition, it has been reported that TβR-II interact with AP2 (Yao et al., 2002). Thus, it is likely that endocytosis of TβR-II receptors is mediated by AP2. However, no dileucine motifs or other internalization signals have been found in type I receptors. Therefore, how type I receptors internalize remain elusive, although a

weak interaction between TβR-I and AP2 has been reported (Yao et al., 2002). Interestingly, it has been documented that TβR-I carrying multiple amino acid mutations within a region, referred to as NANDOR box (non-activating-non-down-regulating), near the carboxyl terminus fail to internalize and are unable to transduce signals (Garamszegi et al., 2001). Therefore, the NANDOR box plays a role in TβR-I endocytosis and might be actively involved in endocytosis of receptor complexes. TβRs internalize into both caveolin- and early endosomal antigen (EEA)1-positive vesicles and reside in both lipid raft and non-raft membrane domains. Clathrin-dependent internalization into the EEA1-positive endosome, where the Smad anchor SARA is enriched, promotes TGF-β signaling. In contrast, the lipid raft-caveolar internalization pathway contains the Smad7-Smurf2 bound receptor and is required for rapid receptor turnover (Di Guglielmo et al., 2003).

A schematic presentation of a general mechanism of TGF-β and the Smad system is presented as follows in Fig. 6.

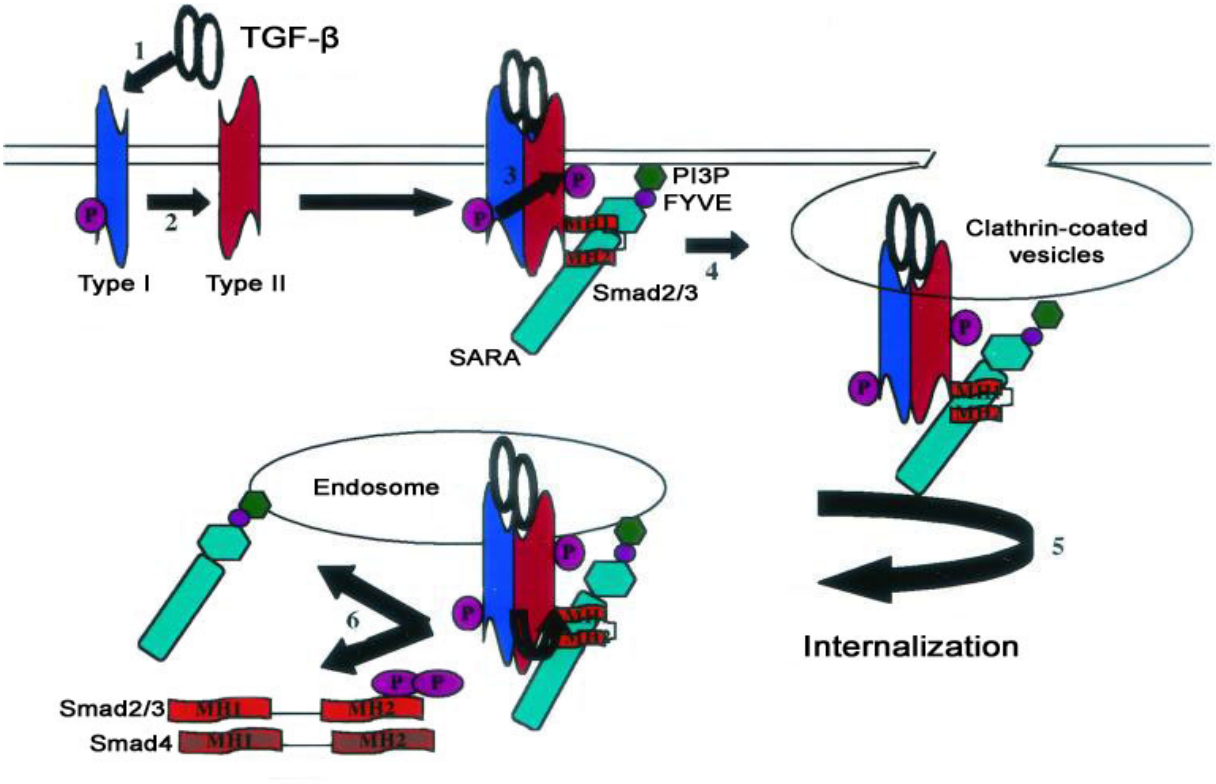

Fig. 6 *General mechanism of TβR and Smad activation. At the cell surface, the ligand binds a complex of transmembrane receptor serine/threonine kinases (types I and II) and induces transphosphorylation in the type I receptor by the type II receptor kinases. The consequently activated type I receptors recruit the FYVE-protein SARA and clathrin-endoytosis occurs. In the endosome, R-Smads are activated and are able to translocate to the nucleus to promote signalling.*

1.3. Chemotaxis

The motility mechanism is ancient, with key molecular components functionally conserved between protozoa and vertebrates. *Chemotaxis* is a general term used to describe the directed cell locomotion in concentration gradients of soluble extracellular agents. Substances that induce a chemotactic response, chemotactic factors, are also known under the general names of cytotaxin, chemotaxin or chemoattractants. Cells showing positive chemotaxis move towards areas with higher concentrations of these agents, those showing negative chemotaxis move away from these areas, i.e. Semaphorins, a class of chemorepellants active in the nervous system (Miao and Klagsbrun, 2000). This behavior is in marked contrast to the reactions elicited by some other compounds, known as *chemokinesis*, which is characterized essentially by an undirected movement of cells, i.e. motogenic cytokines (Timar et al., 2002). The dose-dependent migration of cells induced by gradients of substratum-bound substances has been called *haptotaxis*. *Chemoinvasion* is the migration through a layer of reconstituted basement membrane. *Chemotactic deactivation* is a phenomenon in which leucocytes exposed *in vitro* to a chemotactic factor in the absence of a concentration gradient are rendered relatively unresponsive to stimulation by a subsequent chemotactic gradient. This phenomenon may be a physiologic control mechanism enabling responding cells to remain at sites of the first chemotactic stimulus. The process involves down modulation of receptors (Nelson et al., 1981).

Chemotactically active factors do not constitute a homogeneous class of compounds and include such diverse substances as component C5a of the complement system, N-formylated oligopeptides of bacterial origin, intermediates of lipid metabolism such as arachidonic acid and leukotriene B4 (LTB4), and many cytokines (Lee et al., 2006a) (Chiang et al., 2006) (Lundeen et al., 2006). The chemokines are particularly well known for their chemotactic activities. Chemotactically active factors are active in the nano- to picomole region. They are important mediators of a number of physiological but also pathological processes including, for example, development, embryogenesis, immune responses, inflammation, wound healing, and general systemic reactions following tissue or organ injuries, atherosclerosis, angiogenesis, and metastasis (Anand-Apte et al., 1997). Most of the chemotactically active

cytokines that lead to a local accumulation of cells at the site of secretion merely influence the migration of cells at low concentrations. At higher concentrations (10-100-fold) these cytokines also induce cell activation, a series of co-ordinated biochemical processes that alter, among other things, ion fluxes through the cellular membrane, remodelling of the cytoskeleton, alterations in lipid metabolism, activation of protein kinases.

The biological activity of chemotactically active factors, like that of growth factors or cytokines, is mediated by specific cell surface receptors, which expression can be modulated positively or negatively by almost all cytokines.

Several recent studies suggest that S1PR contribute to cell motility during development (Kupperman et al., 2000). Early studies showed that S1P inhibited migration of several cell types including melanoma, fibrosarcoma, and breast cancer cells (Sadahira et al., 1992) (Wang et al., 1999). In contrast, S1P potently stimulates motility of epidermal cells (Sauer et al., 2004a), immature langerhans cells (Radeke et al., 2005), endothelial cells (Okamoto et al., 2000b) (English et al., 2000), some glioma cells (Van Brocklyn et al., 2003), and, at low concentrations, T cells (Graeler and Goetzl, 2002). In some cases, S1P enhances vascular smooth-muscle cell migration (Boguslawski et al., 2002), while inhibiting movement of these cells in others (Ryu et al., 2002). Overexpression of $S1P_1$ and $S1P_3$ is associated with an increase in the migratory activity (Yamaguchi et al., 2003), while overexpression of $S1P_2$ may cause inhibition of motility or exhibit no effect (Lepley et al., 2005). Inhibition of Gi signaling by using PTX allows S1P to inhibit migration through $S1P_3$ activation of G12/13 (Kimura et al., 2000) (Sugimoto et al., 2003) (Yamashita et al., 2005). $S1P_2$ preferentially couples to G12/13 to inhibit Rac and cell migration. This negative response was shown to be downstream of Rho (Sugimoto et al., 2003). $S1P_4$ may also be able to stimulate migration. Overexpression of $S1P_4$ allowed S1P to stimulate motility through activation of the small GTPase Cdc42 (Kohno et al., 2003). Despite diversity, almost all of the S1PRs have been implicated in regulation (positive or negative) of cell motility.

Motile cells extend a leading edge by assembling a branched network of actin filaments that produces physical force as the polymers grow beneath the plasma membrane (Pollard and Borisy, 2003). Almost universally, crawling motility involves a cycle of four steps: protrusion of the leading edge, adhesion to the substratum, retraction of the rear, and de-adhesion (Pollard and Borisy, 2003). When cells move,

they use actin polymerization to push the plasma membrane outward. Protrusive structures formed by migrating cells were termed filopodia, lamellipodia, and invadopodia/podosomes, dependently on their morphological, structural, and functional characters (Fig. 7).

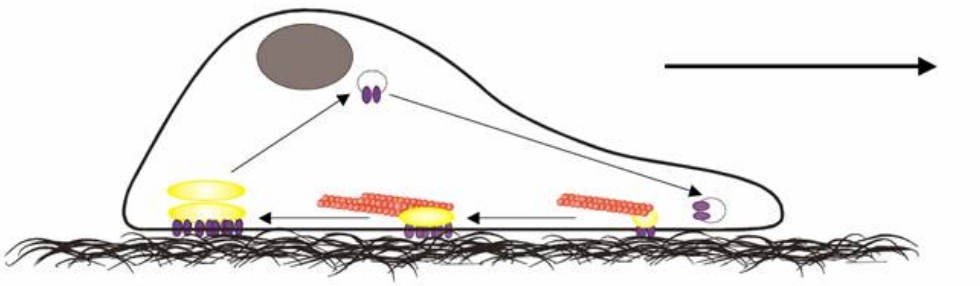

Fig. 7 *The four-step motility cycle. Cells migrating need to form new membrane protrusions, lamellipodia, at the leading edge. Formation of these structures is driven by localized actin polymerization (red). The adhesion to the substratum requires new focal adhesions (violet). Retraction of the rear and de-adhesion move the cell in the direction of the arrow.*

Lamellipodia are flat, sheet-like membrane protrusions formed at the leading edge of migrating cells. It is generally believed that lamellipodia have a major role in driving cell migration by attaching to the substrate and generating force to pull the cell body forward. Lamellipodia contain dendritic arrays of actin filaments and the molecular machinery that controls polymerization/depolymerization and organization of actin filaments (Nicholson-Dykstra et al., 2005). Filopodia are thin, finger-like projections consisting of bundled, crosslinked actin filaments. They are also observed at the migrating front of cells (Faix and Rottner, 2006). The protrusion of lamellipodia requires generation of free-barbed ends of actin filaments at the leading edge. There are three major mechanisms for generation of free barbed ends: (1) *de novo* nucleation by Arp2/3 complex and formins, (2) severing of pre-existing actin filaments by cofilin, and (3) uncapping of barbed ends on pre-existing actin filaments (Zigmond, 2004) (Condeelis, 2001). A model proposed for migration is next described in detail.

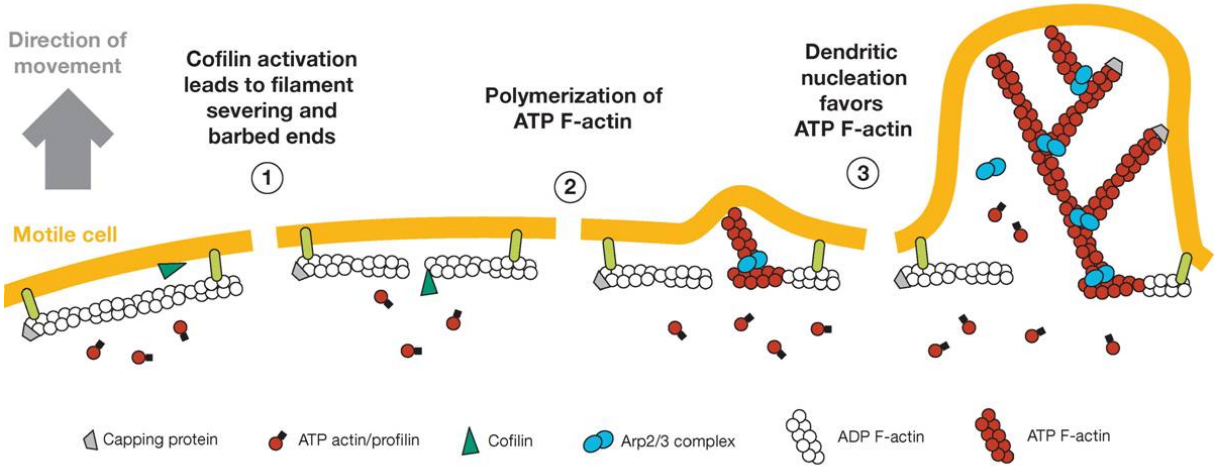

Fig. 8 *The stimulated protrusion model showing the role of cofilin severing in determining the site of dendritic nucleation, protrusion, and cell direction. Severing of actin filaments in the cortical actin cytoskeleton by cofilin creates free barbed ends (1) that bias the location and the amount of dendritic nucleation by the Arp2/3 complex. Polymerization (2) proceeds from a pool of pre-existing actin monomers, allowing the initiation of polymerization to occur without being tightly coupled to depolymerization. The dendritic nucleation activity of the Arp2/3 complex forms daughter filaments (3).*

Unstimulated cells have non-polarized cell morphology, in which the molecular machinery for generation of free barbed ends is inactive. Chemoattractant stimulation induces a rapid dephosphorylation and subsequent local activation of cofilin at the leading edge, which leads to severing of pre-existing actin filaments and formation of new free barbed ends from which new actin filaments are assembled. This initiates membrane protrusions and sets the direction of cell migration. Simultaneously, WASP (Wiskott-Aldrich syndrome protein) family proteins integrate upstream signals as well to induce actin polymerization through their downstream effector complex Arp2/3, an activator of actin filament nucleation and network branching (Millard et al., 2004). Afterwards, the branched actin filaments are stabilized by cortactin. This strengthens the protrusive force of lamellipodia and leads to cell movement (Yamaguchi and Condeelis, 2006). Many chemotactic factors have been shown to stimulate intracellular signaling pathways, which lead to barbed end formation through these mechanisms (Xue et al., 2006). S1P mediated actin polymerization at and distribution of Arp2/3 complex as well as endogenous cortactin into the cell-leading edge has been recently described in endothelial cells (Lin et al., 2005). Furthermore, a novel molecular mechanism was proposed involving Gi-mediated Akt-

dependent $S1P_1$ receptor phosphorylation and Cdc42/Rac activation pathways to promote cortactin-Arp2/3 complex formation (Lee et al., 2006b).

Proteins from the Rho family small GTPases, are considered as central regulators of protrusions involved in actin organisation and formation of lamellipodia and filopodia. Cdc42 regulates polarity of eukaryotic cells and is active toward the front of migrating cells (Itoh et al., 2002b). Besides Rho-GTPases PI3K, integrins, microtubules and vesicular transport also contribute to the establishment and maintaining of cell polarity in response to an extracellular signal. Thus, PI3Ks accumulate to the leading edge of the cell downstream of a chemoattractant signal, whereas the corresponding phosphatase PTEN localises at the sides and the rear (Devreotes and Janetopoulos, 2003; Merlot and Firtel, 2003). Rac is involved in initiating and stabilising protrusions in a very complex manner by interacting with phosphoinositol-3-kinases (PI3Ks) (Srinivasan et al., 2003; Welch et al., 2003), integrins (del Pozo et al., 2000; Kiosses et al., 2001; Schwartz and Shattil, 2000) and microtubules (Rodriguez et al., 2003). By suppressing the other's activity Rho and Rac are mutually antagonistic (Evers et al., 2000) thereby suppressing Rho effects at the cell front and Rac induced formation of protrusions at the sites (Worthylake and Burridge, 2003; Xu et al., 2003a).

Adhesion proteins, including cadherins, fibronectin, vitronectin and laminin, which are part of the ECM, play an important role in the mechanism of cell motility. These molecules support the formation of attachments of the protrusions to the ECM, termed adhesions plaques, at the front of the cell to provide stable traction. These attachments are realised by cell-surface located receptors, known as integrins (Mizejewski, 1999). Integrins transmit force from the actin cytoskeleton to the ECM, thus establishing traction sites for migration (traction stage) (Felsenfeld et al., 1996; Felsenfeld et al., 1999) and they transmit information about the quality of the ECM resulting in cytoskeletal changes (Beningo et al., 2001; Galbraith et al., 2002; Lauffenburger and Horwitz, 1996). After integrin mediated cell attachment Src interacts with focal-adhesion proteins, including focal-adhesion kinase (FAK) and the docking proteins p130Cas (Cas), Crk and paxillin to form focal adhesions at the leading edge. This interaction influences Src-dependent integrin-cytoskeleton interactions and is required for migration (Cary et al., 1998; Ilic et al., 1995; Klemke et al., 1998). Src signalling and therefore integrin-cytoskeleton interactions are affected by Rho-GTPases, Rac and Cdc42, mediating extracellular signals. Adhesion

complexes are specialized structures that include a large number of cytoskeletal proteins. Such molecules include vinculin, paxillin, and FAK (FA kinase). Paxillin is a FA adaptor protein that transduces integrin and growth factor signalling from the cell surface to the actin cytoskeleton. Paxillin is phosphorylated by FAK in response to cell-cell or cell-ECM adhesion and subsequently binds several proteins (i.e., vinculin) that contribute to the organization of actin cytoskeleton at the focal adhesion sites. Cells lacking vinculin are highly motile by controlling the accessibility of paxillin for FAK interaction (Subauste et al., 2004). Vinculin contains a paxillin binding site in that way being able to modulate paxillin-FAK interactions. Hence, vinculin indirectly inibits FAK interactions leading to stress fiber formation, focal adhesion, and subsequently decrease in motility and therefore, is implicated in FA turnover (Turner, 2000). Adhesion turnover constitute the final stage of motility by loosening the linkages at the cell rear promoting tail retraction as well as at the base of protrusions at the leading edge along with the formation of new protrusions (Webb et al., 2002). Adhesion disassembling is regulated by FAK-Src interaction together with Cas and Crk via Rac depending pathways (Turner et al., 2001).

In different cell types, the details of the migration process differ depending on cell environment and nature. However generally, cell movement is orchestrated by the complex interplay of actin network formation at the leading edge and continuous formation and disassembly of focal adhesions.

1.4. Physiological relevance of S1P-induced motility in human fibroblasts

Connective tissue, derived from mesoderm, the middle germ cell layer in the embryo, is a material made up of fibers forming a framework and support structure for body tissues and organs. These tissues form a matrix for the body. The functions of connective tissues are varied. They are largely responsible for the cohesion of the body as an organism, of organs as functioning units, and of tissues as structural systems. The connective tissues are essential for the protection of the body both in the elaborate defence mechanisms against infection and in repair from chemical or physical injuries. Connective tissues are important in the development and growth of many structures. Constituting the major environment of most cells, they are probably the major contributor to the homeostatic mechanisms of the body. The connective tissues consist of cells and extracellular or intercellular substance (Fig. 9). The cells include many varieties, of which the following are the most important: fibroblasts, macrophages (histiocytes), mast cells, plasma cells, melanocytes, and fat cells.

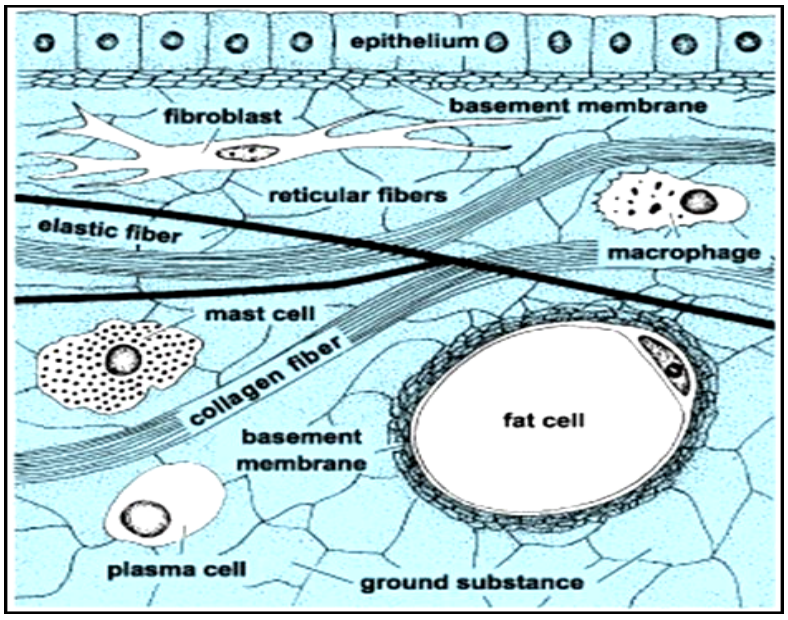

Fig. 9 *Components of connective tissue.*

Most of the cells of the connective tissue are developmentally related even in the adult; for example, fibroblasts may be developed from histiocytes or from undifferentiated mesenchymal cells. The extracellular components of connective tissues may be fibrillar with two major structural proteins, collagen and elastin or nonfibrillar, in which cells and fibers are embedded. In addition, the ground substance contains metabolites derived from, or destined for, the blood.

Injury to connective tissue involves damage to the cells and structural components of the tissue. Several responses are triggered and a sequence of events begins to repair the tissue. The reaction to injury includes vascular, cellular, and biochemical responses. Three phases of the repair process can be applied to the general healing of connective tissue: acute inflammation phase, matrix and cellular proliferation phase, and remodelling phase. Efficient cutaneous wound repair is initiated by hemostasis and inflammation that results in the formation of a scar, which partially recapitulates the initial tissue structure and function (Sandulache et al., 2006). Contraction of the wound and synthesis of ECM restore skin integrity. Inflammation begins with a release of chemical mediators from cells into the extracellular fluid. Chemical mediators released by inflammatory cells stimulate migration and proliferation of fibroblasts, which participate in the repair process by contributing to the formation of a new granulation tissue (Singer and Clark, 1999) and generate traction to effect wound closure (Clark, 1993). Human fibroblasts react to new serum factors that are absent in plasma, with proliferation to expand, migration into the wound bed, synthesis of new ECM, and differentation in myofibroblasts (Singer and Clark, 1999). A number of growth factors/cytokines have been reported to affect, directly or indirectly, fibroblasts motility and would provide a potent signal for fibroblast migration into the wound bed. They include basic and acidic FGF (Fibroblast Growth Factor), TGF-β 1 and 2, VEGF, PDGF, EGF (epidermal growth factor), and IL-1 (interleukin-1) (Vogt et al., 1998) (Imanishi et al., 2000) (Sillman et al., 2003). Comparable to TGF-β, the lipid mediator S1P is secreted by platelets upon activation (Yatomi et al., 1995) (Tani et al., 2005) at wound sites and has a broad spectrum of effects on, and is secreted by, each of the diverse cell types involved in wound healing. Enzymes that participate in S1P metabolism, such as SphK, are tightly regulated by pro-inflammatory factors like the tumor necrosis factor (TNF) and IL1 (Pettus et al., 2005). TGF-β is considered as a pro-fibrotic growth factor by stimulating matrix deposition through modulation of fibroblast recruitment and

proliferation (Konttinen et al., 2000). Migrated fibroblasts (normally quiescent) start to proliferate, change their phenotype into myofibroblasts, thus becoming the predominant source of increased collagen production, and synthesize a wide range of mediators that noticeably affect fibroblasts proliferation, differentiation, and migration. Impairment of fibroblasts modulatory activity might lead to the self-perpetuation of the reparative process and eventually to fibrosis, which is characterised by the development of excess fibrous connective tissue (scar tissue) in an organ after injury.

Connective tissue cells such as dermal fibroblasts play an important role in many (patho)physiological conditions such as scurvy, systemic lupus erythematosus, rheumatoid arthritis, scleroderma, fibrotic disorders, and the controversial wound healing processes. Some pathological situations derived from an impaired regulation of fibroblasts motility at wound sites are resumed in the following.

Chronic venous insufficiency causes characteristic changes, called lipodermatosclerosis, to the skin of the lower extremities, which leads to eventual skin ulceration. Venous ulceration is the most severe and debilitating outcome of chronic venous insufficiency. It is characterised by dermal changes and impaired venous ulcer healing. Dermal fibroblasts show an altered ability to migrate due to chronic wound fluid from venous ulcers (Raffetto et al., 2001).

Lupus is a condition of chronic inflammation caused by an autoimmune disease. Sometimes lupus can cause disease of the skin, heart, lungs, kidneys, joints, and/or nervous system. When only the skin is involved, the condition is named discoid lupus. When internal organs are involved, the condition is called systemic lupus erythematosus (SLE). Cutaneous LE is a nonscarring non-atrophy-producing photosensitive dermatosis.

Scleroderma is an autoimmune disease of the connective tissue. Scleroderma is characterized by the formation of scar tissue (fibrosis) in the skin and organs of the body. This leads to thickness and firmness of involved areas. Scleroderma is also referred to as systemic sclerosis. The diffuse form of scleroderma is involved in symmetric thickening of skin of the extremities, face, trunk (chest, back, abdomen, or flanks), which can rapidly progress to hardening after an early inflammatory phase. Organ disease can occur early on and be serious. Organs affected include the esophagus, bowels, lungs with scarring (fibrosis), heart, and kidneys. It has been reported that activated fibroblasts, which have previously been obtained from

bronchoalveolar lavage fluid (BALF), are proposed to be important cells in the fibrotic processes of scleroderma. The presence of these cells in the airway lumen occurs after motility of BALF-derived fibroblasts in patients with scleroderma (Larsen et al., 2006).

Rheumatoid arthritis is a chronic autoimmune/inflammatory disease which leads to progressive joint damage and destruction (Brennan et al., 1998). Fibroblast-like synoviocytes play a major role in the pathogenesis of rheumatoid arthritis by secreting effector molecules that promote inflammation and joint destruction. IL-1 and TNF are the principal cytokines occurring in most of these acute and chronic inflammatory diseases (Chicheportiche et al., 2002). They are mainly produced by macrophages, however it has been reported that also skin fibroblasts accumulate intracellular pre-IL-1 leading to the final release of IL-1 (Kawaguchi et al., 2006).

Modulation of both fibrotic and inflammatory processes and cellular signals underlying such events are not fully understood. Hence, regulation of fibroblast migration is not straightforward; the effect may be indirect or dependent on cytokine concentration and network. Some cytokines and/or growth factors act as competing rather than progression factors, some lack secretory signals, and some must be processed and released from the pericellular matrix or basement membranes (i.e. TGF-β). Comprehension of how human dermal fibroblasts react to serum-released mediators, such as S1P, which as well as TGF-β, is secreted in the connective tissue and hold pro-fibrotic and -angiogenic properties, may help to find therapeuticall agents against pathophysiological conditions.

1.5. Aim of the work

Cell migration is required for many biological processes, such as embryonic morphogenesis, immune surveillance, and tissue repair and regeneration. Aberrant regulation of cell migration drives progression of many diseases, including cancer invasion, and metastasis (Condeelis et al., 2005) (Sahai, 2005). Therefore, understanding the fundamental mechanisms of cell migration is critical for our understanding of both basic biology and the pathology of disease. The lysophospholipoid S1P is a controversial molecule with pleiotropic properties and signaling inside and outside the cell. S1P is a lipid mediator that has novel dual actions, that still have to be elucidated. Hence, S1P shows positive and negative effects on many of its responses. Regarding migration, this lipid acts as a strong chemoattractant for a variety of cells as well as a potent antimigratoric mediator. However, the effect of S1P on the migration of primary dermal fibroblasts has not been investigated yet. In order to elucidate the chemotactic properties of S1P, this work was aimed to the directed migration or chemotaxis on fibroblasts. Understanding the molecular basis for the regulation of dermal cell migration could help develop a novel way of preventing many forms of lesions, in which they are involved.

Many signaling pathways are activated through S1P to induce migration, but the fact that blocking them, through antisense techniques or specific inhibitors, still produces motility indicate that other still unknown signaling pathways must be implicated. Thus, the dual effects of S1P on motility depending on cell type, receptor expression or G protein associated reveals a versatile chemoattractant, extremely conservative regulated, which response to external stimuli is still unpredictable. It is well known that GPCRs cross-communicate with other receptors to signal, but it has been described recently that different G proteins can also crosstalk within the same receptor. Furthermore, the idea that GPCRs act as heteromers rather than as monomers, and trans-activation within G proteins as well as with other receptor systems, is becoming more acceptable. As seen, the molecular mechanism underlying S1P responses remain unclear.

In this work, migration of dermal fibroblasts towards S1P, the novel connection to the TGF-β-Smad system, and the receptors involved in those processes have been

investigated. Furthermore, evidence for the signaling mechanism of S1P leading to fibroblasts migration has been given. For this purpose, a possible crosstalk with the serine/threonine TGF-β receptor has been researched.