UNIVERSITÄT
BERN

Graduate School for Cellular and Biomedical Sciences

University of Bern

The role of the $S1P_3$ receptor in inflammation-associated proliferative disorders

PhD Thesis submitted by

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for the degree of

PhD in Biomedical Sciences

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Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

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ABSTRACT

Inflammation is involved in the pathogenesis of many different disorders, including glomerulonephritis, fibrosis and cancer. Unraveling the regulatory mechanisms underlying the molecular processes of inflammation remains crucial for the development of novel therapeutics for future treatment of inflammatory disorders.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid molecule, which likely plays an important role in inflammation. However, the molecular mechanisms involved in S1P-mediated inflammation are still not completely understood. This work aimed to unravel the interconnection between S1P and other molecular players implicated in inflammatory response, as well as to indicate the S1P receptor subtype the activation of which contributes to proliferation and migration of non-cancerous cells and to metastatic progression of breast cancer. In addition, the therapeutic potential of drugs targeting early steps of the inflammatory response was assessed.

As model systems for the non-cancerous studies we used renal mesangial cells (rMC) and mouse embryonic fibroblasts (MEF), and for the cancer studies the breast cancer cell line MDA-MB-231 and two metastatic sublines derived from lung (4175) and bone (1833).

Our data demonstrate that the $S1P_3$ receptor is strongly upregulated in lung and bone metastatic cell lines compared to the parental MDA-MB-231 cells, and its activation by S1P has pro-inflammatory, pro-migratory and pro-invasive potential by inducing COX-2 expression and PGE_2 signaling via EP_2 and EP_4. These data were confirmed in non-cancerous studies, where we found that $S1P_3$ receptor is upregulated in mMC and MEFs upon the loss of SphK2. Our results show that the deficiency of SphK2 in these cells correlates with an enhanced proliferative and migratory capacity, indicating that SphK2 exerts suppressive effects on cell growth and migration. This finding is likely to be due

to the enhanced expression of $S1P_3$, as its depletion in mesangial cells reduced their proliferation and migration rate.

Our next study revealed that the novel $cPLA_2$ inhibitors AVX001 and AVX002 have an anti-inflammatory potential in cultures of rMC by reducing the pro-inflammatory mediator PGE_2 through the inhibitory effect on NFκB activation. Thus, they represent promising novel drugs for the treatment of inflammatory disorders.

Altogether, our data demonstrate for the first time a critical role of the $S1P_3$ receptor in S1P-mediated proliferative and inflammatory responses, and identify $S1P_3$ as a potential target for therapeutic intervention to treat inflammation-associated proliferative disorders.

ABBREVIATIONS

AA	arachidonic acid
ABC	ATP-binding cassette
Akt/PKB	protein kinase B
BSA	bovine serum albumin
C1P	ceramide 1-phosphate
CNS	central nervous system
COX	cyclooxygenase
CTGF	connective tissue growth factor
DMEM	Dulbecco's modified Eagle's medium
EDG	endothelial differentiation genes
EGFR	epidermal growth factor receptor
EP	E prostanoid receptors
ER	endoplasmic reticulum
$ER^{+/-}$	estrogen receptor positive/negative
GN	glomerulonephritis
ERK	extracellular regulated kinase
G-protein	guaninenucleotide binding proteins
GPCRs	G-protein-coupled receptors
IL-1β	interleukin-1β
IP3	inositol triphosphate
IUPHAR	International Union of Basic and Clinical Pharmacology
JNK	c-jun N-terminal kinases
kDa	kilo Dalton
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase

MEFS	mouse embryonic fibroblasts
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PAI-1	plasminogen-activator inhibitor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PGs	prostaglandins
PGE_2	prostaglandin E_2
PKC	protein kinase C
$cPLA_2$	cytosolic phospholipase A_2
$iPLA_2$	calcium-independent phospholipase A_2
$sPLA_2$	secretory phospholipase A_2
PLC	phospholipase C
cPGES	cytosolic prostaglandin E synthase
mPGES	microsomal prostaglandin E synthase
SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electrophoresis
S1P	sphingosine-1 phosphate
S1PRs	sphingosine-1 phosphate receptors
SM	sphingomyelin
SMases	sphingomyelinases
SPAK	stress-activated protein kinase signaling complex
SphK (SK)	sphingosine kinase
STAT3	signal transducer and activator of transcription 3
TNF-α	tissue necrosis factor α
uPAR	urokinase-type plasminogen activator receptor

Chapter 1

GENERAL INTRODUCTION

1.1. Introduction to sphingolipids

Sphingolipids were first time described in 1884 by the German surgeon Johann Ludwig Wilhelm Thudichum who isolated a variety of novel lipid species from brain extracts. Due to the enigmatic nature and the riddle of the functions, the smallest molecule in this group was named "sphingosine" after the Sphinx in Greek mythology [1]. The broader term *"sphingolipids"* was proposed later by Carter et al. [2] for all lipids which derive from sphingosine. Over the last century, a large number of sphingolipid subspecies have been isolated and characterized.

1.1.1. Chemical structure of sphingolipids

Sphingolipids are amphipathic molecules with both hydrophobic and hydrophilic properties. The hydrophobic part is represented by the sphingoid base, a long-chain amino alcohol of 18 – 20 carbon atoms, and a fatty acid coupled through an amide bond at C2 position, whereas the hydrophilic part is represented by the head group. The diversity of sphingolipid species is the result of structural variations in the backbones, in the attached fatty acids and the head groups (Fig. 1).

Sphingosine (sphinganine) is the prevalent backbone of all mammalian sphingolipids, although various other long-chain bases are also known, mainly characterized by different alkyl chain lengths, positions of double bonds (sphingadienes), degrees of saturation (the saturated form of sphingosine is sphinganine) and degrees of hydroxylation (phytosphingosine). Free sphingoid bases are typically present in very small amounts, most of them are amide-linked to fatty acid [3].

The N-acetylated sphingoid bases lacking additional head groups, generally termed *"ceramides"*, are precursors for all complex sphingolipids. Depending on the backbone, ceramides are subclassified into dihydroceramide

(derivatized from sphinganine), ceramide (backbone – sphingosine) or phytoceramide (backbone – phytosphingosine). Fatty acid chains are usually 14 – 36 carbon atoms in length and saturated, with a single double bond or a hydroxyl group, although there are also many variations of acyl chain composition. Ceramide plays an important role as a signaling molecule or can be converted to higher sphingolipids by addition of a hydrophilic head group at the sphingoid C1 hydroxyl group [4,5]. For instance, the transfer of phosphocholine yields *sphingomyelin* (SM) by the action of SM synthase, or the

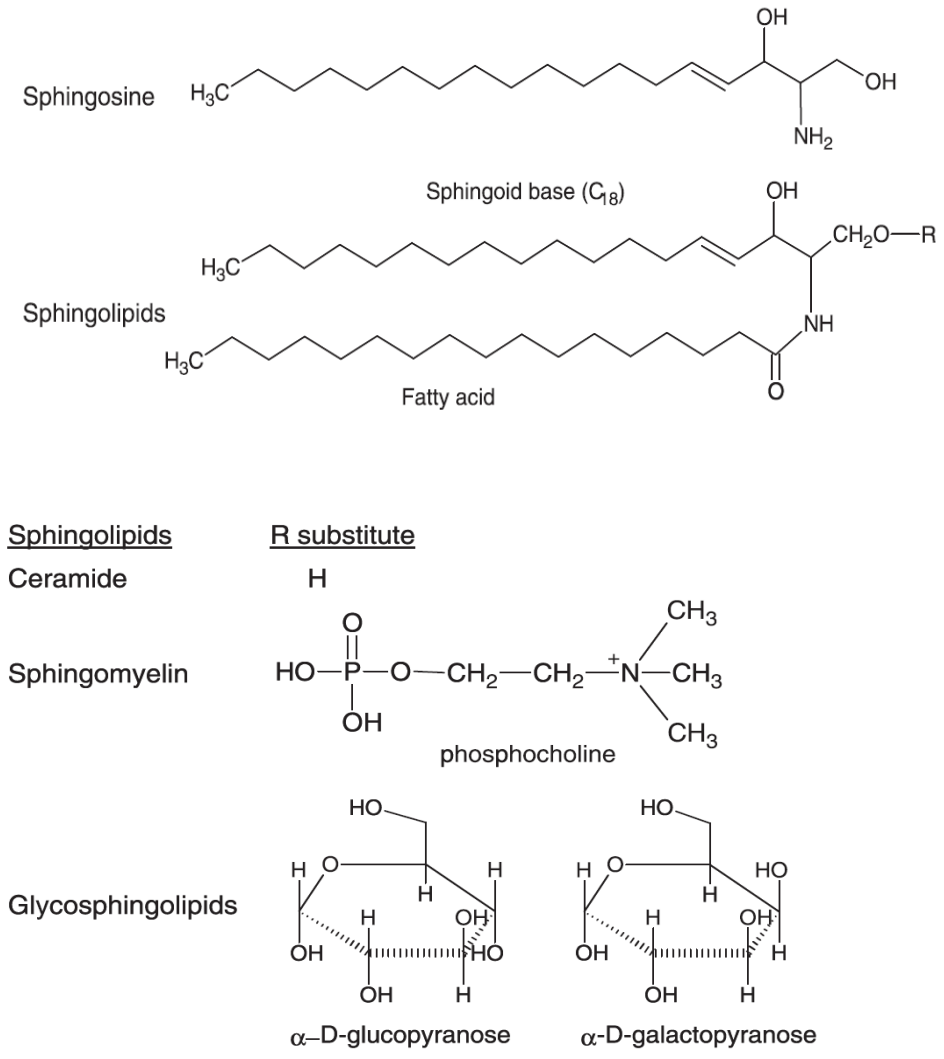

Fig. 1. Basic structure and classification of sphingolipids [6].

transfer of sugar residues to yield *glycosphingolipids*, including gangliosides and sulfatides [7].

There is also a small number of *lyso-sphingolipids* existing, such as sphingosine 1-phosphate (S1P), sphingosine 1-phosphocholine (lysosphingomyelin) and lyso-glycosphingolipids. Of these lyso-sphingolipids, only S1P has been extensively characterized so far. The functions or regulations of lysosphingomyelin and lyso-glycosphingolipids are still poorly understood.

1.1.2. The sphingolipid biosynthesis

The generation and degradation of sphingolipids is regulated by interconnected metabolic pathways. All sphingolipids originate from the same *de novo* synthesis pathway starting at the cytosolic leaflet of the endoplasmic reticulum with the condensation of L-serine and palmitoyl CoA by action of serine palmitoyltransferase, and yielding 3-keto-dihydrosphingosine [8,9]. In turn, 3-keto-dihydrosphingosine is reduced to dihydrosphingosine (sphinganine) and subsequently acylated by (dihydro) ceramide synthases resulting in the production of dihydroceramide [10]. Ceramides are formed by desaturation of dihydroceramide [11] and can further be either phosphorylated by ceramide kinase to C1P [12], glycosylated by glucosyl- or galactosyl ceramide synthases to form glycosphingolipids [13] or converted back to SM by the action of SM synthases [14].

The breakdown of complex sphingolipids back to ceramide takes place in the lysosomes and is catalyzed by specific hydrolases, which all possess a pH optimum in the acidic range. The final steps are carried out by a glucosidase from glucosylceramide, galactosidase from galactosylceramide or an acid SMase from SM [15]. Ceramide can be further deacetylated by a lysosomal acid ceramidase leading to the formation of sphingosine [16]. Sphingosine is the only species which is able to exit the lysosome and thereby can feed into the *de novo* pathway or serve as a substrate for two sphingosine kinases (SphKs) [17].

The product, sphingosine 1-phosphate (S1P), can be converted back to sphingosine by the action of specific S1P phosphatases (SPP1 and SPP2) and unspecific phosphate phosphatases [18,19]. It can also be irreversibly degraded by the enzyme S1P lyase to non-sphingolipid products, phosphoethanolamine and hexadecenal [20].

Sphingolipids are essential components of eukaryotic cell membranes and comprise ~ 10 – 20% of membrane lipids, thereby executing important structural roles in the regulation of the membrane fluidity and subdomain structure of the lipid bilayer [5]. Furthermore, a multitude of studies on sphingolipid metabolism and action have been performed during the last decades now revealing that certain subspecies, most importantly ceramide, sphingosine, S1P and C1P, also act as intercellular and intracellular signaling molecules.

1.1.3. The cellular sphingolipid rheostat

Ceramide, sphingosine and S1P are considered as critical mediators of cell survival and death. Based on the findings that ceramide and sphingosine induce cell growth arrest and apoptosis [21], whereas S1P rather mediates cell proliferation and survival [22], and that these two molecules are inter-convertible within the cells, the term “sphingolipid rheostat” was proposed [23]. According to this concept, the dynamic balance between ceramide/sphingosine and S1P regulates the cell’s fate in response to different stimuli [24,25].

Many studies so far have presented evidence that ceramide is an important second messenger in various stress responses and plays a crucial role in cell death [21,26,27]. Different stress stimuli like TNFα, γ-interferon, interleukin-1β, UV light, doxorubicin, etc., induce the formation of ceramide through the activation of either SMase or *de novo* synthesis [27,28]. In turn, ceramide can directly affect a variety of enzymes involved in stress signaling cascades, including protein kinase C (PKC) isoenzymes [29,30], c-Raf [31], cPLA2 [32],

KSR [33], cathepsin D [34] and protein phosphatases (PP1 and PPA2) [35,36]. It has also been shown that ceramide can induce apoptosis in cells by the mitochondrial pathway [37]. For instance, ceramide-associated inhibition of the PI3K/Akt pathway and ceramide-induced activation of cathepsin D leads to dephosphorylation of pro-apoptotic Bcl-2 family members Bad and Bid, respectively, and activation of apoptotic pathways in mitochondria [34,38]. Therefore, ceramide accumulation mediates mitochondrial outer membrane permeabilization by regulating different Bcl-2 members by various mechanisms.

Sphingosine has been attributed both growth promoting and growth inhibiting or apoptotic effects, which seems to depend on the cellular activity of SphKs to convert sphingosine to the growth promoting S1P or on a direct effect of sphingosine's molecular targets, such as PKC isoenzymes [39]. However, the detailed mechanisms of sphingosine-mediated cell death are still unclear. A recent study showed that sphingosine interacts with the pro-survival adaptor protein 14-3-3 and allows phosphorylation of this molecule by protein kinase A (PKA) and PKCδ, therefore blocking an important pro-survival pathway [40].

The effects of ceramide and sphingosine could be attenuated by an increase of S1P production, which has been associated with cell proliferation and survival [22,23,41]. Therefore, the agents that regulate the conversion of ceramide/sphingosine to S1P may play a crucial role in cell fate. Two isoforms of SphKs, which catalyze the synthesis of S1P from sphingosine and denoted SphK1 and SphK2, have been discovered and partially characterized [42,43]. Although both of these enzymes use the same substrate to produce the same product, they have different subcellular localizations and are hypothesized to exert differential cellular activity. For example, SphK1 promotes cell growth, whereas SphK2 rather triggers cell death. SphK1, but not SphK2, has emerged as a critical regulator of the sphingolipid rheostat. Indeed, as a consequence of SphK1 inhibition, not only cellular S1P levels decrease, but also cellular

ceramide levels increase, which may then reprogram cells towards cell death [44].

Given this key role of sphingolipids in regulating cell proliferation and survival, it has been suggested that the sphingolipid rheostat is dysregulated in proliferative disorders, including cancer, glomerulonephritis, and fibrosis. In these diseases, SphK1 was shown to be upregulated and to overproduce the pro-survival factor S1P [45–48].

1.1.4. Sphingosine 1-phosphate – a specific ligand for G-protein coupled receptors

Sphingosine 1-phosphate (2*S*-amino-1-(dihydrogen phosphate)-4E-octadecene-1,3*R*-diol) is a key bioactive molecule which regulates various biological and pathophysiological cellular responses (Fig. 2) [49]. Many studies point to an intracellular role for S1P, where it acts as a second messenger by binding to several proteins and regulating their functions. For instance, it has recently been suggested that nuclei contain a high amount of S1P, which regulates gene transcription by inhibiting histone deacetylases [50]. In addition, S1P can act as a missing cofactor for activation of NF-κB pathway [51], mediate cytochrome-c oxidase assembly and respiration in mitochondria and induce cellular inhibitor of apoptosis (cIAP2) [52]. However, the majority of important biological effects are known to be mediated by extracellular S1P. Accumulating evidence now suggests a contribution of extracellular S1P to diseases associated with inflammation, proliferation and resistance to apoptosis [53–56], which makes it an attractive target in biomedical research.

The concentration of S1P in the blood and lymph plasma is high and reaches micromolar ranges, whereas S1P concentration in tissues is low, creating a S1P gradient [57]. For many years, it was assumed that blood platelets represent the main source of plasma S1P [58]. However, clear new data

have recently been reported that erythrocytes and, to some extent, also endothelial cells are the major sources of plasma S1P [59,60].

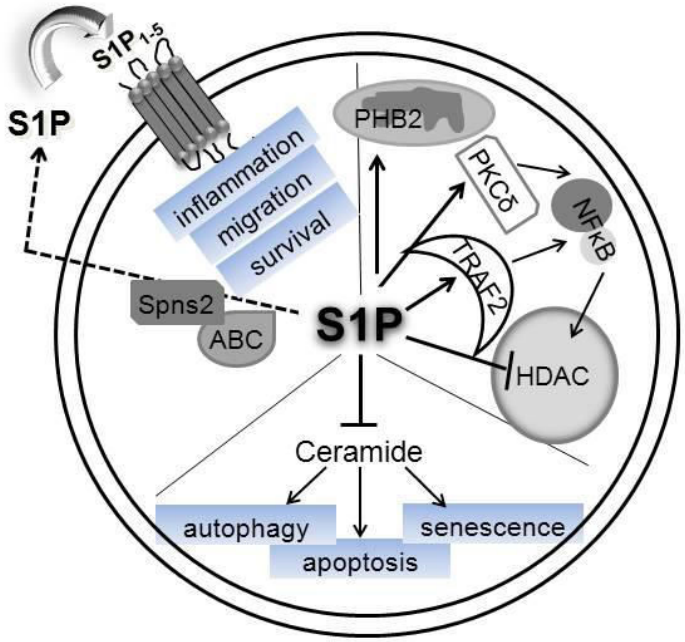

Fig. 2. Schematic representation of cellular processes regulated by intracellular and extracellular S1P [61].

Little is known about the molecular mechanisms by which S1P is secreted into the extracellular environment. Since S1P has a polar head group and cannot pass the cell membrane, it requires the activity of a transporter system to reach the extracellular space from its intracellular site of synthesis. The ATP-binding cassette (ABC) family of transporters was first identified *in vitro* to be involved in S1P export [62]. However, the relevance of these transporters in S1P export has been questioned, since ABC knockout mice showed no phenotype of S1P distribution, which suggests the existence of other S1P-transporters. Indeed, recently the spinster homolog 2 (Spns2) was recognized as a unique type of S1P transporter [63]. Although, Spns2 appears to be the main S1P exporter in endothelial cells based on the *in vitro* and *in vivo* data, other studies showed that it is not involved in S1P release from platelets and erythrocytes [64], raising the

possibility that Spns2 is not the only regulator of the plasma S1P concentration [65].

Outside the cell S1P acts in an autocrine and paracrine manner as a ligand for specific G protein-coupled receptors, named S1P receptors (S1PRs) [66]. These receptors were originally named endothelial differentiation genes (EDG), based on the first detected member of the family, EDG-1, which was identified *in vitro* as an inducible transcript of endothelial cell differentiation [67]. So far, eight receptor subtypes have been described. Although, the later discovered receptors of this family have no involvement in endothelial differentiation, they were characterized based on their sequence similarity. With the identification of S1P as a high affinity ligand for EDG-1 [68] the classification was formalized. Today the IUPHAR has renamed these EDG receptors to S1P receptors ($S1P_{1-5}$) and LPA receptors (LPA_{1-3}), according to their main physiological ligand and the chronological order of discovery within the family [69].

There are currently five bona fide cognate GPCRs for S1P ($S1P_{1-5}$, formerly, EDG-1, -5, -3, -6, -8 respectively). A specific repertoire of S1P receptors expression in different organs and tissues, and their individual ability to couple different G proteins (guanine nucleotide binding proteins) (α_i, α_q or $\alpha_{12/13}$) with subsequent activation of corresponding downstream signaling pathways designate the diversity of S1P-mediated effects. Over the last years a tremendous progress has been made in understanding the properties and functions of the S1PRs family (Table 1.1.). However, the molecular players involved in S1PRs signaling and the cellular responses triggered by these receptors are still not fully understood. The complexity of S1P binding and activating different S1PR subtypes that couple to a variety of G proteins that finally leads to either additive effects or neutralizing effects, underscore the need for further investigation. Indeed, studies, which address the possible role of S1PRs in the development of different diseases, may provide novel therapeutic targets.

Table 1.1. S1P receptors and their biological effects

Receptor	Main tissue distribution	G protein	Downstream signaling	Cellular responses
$S1P_1$	Ubiquitous	$G_{i/o}$ Rho Rac↑	ERK1/2↑ PI3K/Akt↑ AC↓	Migration↑, proliferation↑, survival↑, cell-cell contacts, angiogenesis↑, lymphocyte trafficking
$S1P_2$	Ubiquitous	$G_{i/o}$ G_q $G_{12/13}$ Rac↓ Rho↑	PLC↑ $[Ca^{2+}]_i$↑ ERK1/2↑ JNK↑ p38↑	Migration↓, contribution to vascular development, pro-apoptotic signaling
$S1P_3$	Ubiquitous	$G_{i/o}$ G_q $G_{12/13}$ Rho↑ Rac↑	PLC↑ $[Ca^{2+}]_i$↑ ERK1/2↑ Akt↑	Heart rate↓, migration↑, proliferation↑, survival↑, contribution to vascular development
$S1P_4$	Lymphoid tissue and hematopoietic cells	$G_{i/o}$ $G_{12/13}$ Rho↑↓	PLC↑ $[Ca^{2+}]_i$↑ ERK1/2↑ Cdc42↑ AC↑	Migration↑↓, cytoskeleton rearrangement
$S1P_5$	CNS, NK cells, spleen and skin	$G_{i/o}$ G_{12}	ERK1/2↓ AC↓ p54↑ JNK↑	Proliferation↓, brain myelinization

1.2. Inflammation

Inflammation is an important cellular response triggered by various mechanical, chemical or immunological stress factors and characterized by symptoms of pain, fever, redness, swelling and loss of function. It represents a complex of events starting with an initial production of pro-inflammatory mediators which recruit professional inflammatory cells to the site of injury to dispose the offending trigger [70]. Inflammation is now recognized as a hallmark of various diseases, including arthritis, kidney disorders (glomerulonephritis, fibrosis, acute kidney injury) and cancer therefore, understanding the inflammation cascade is mandatory for the development of new treatments.

1.2.1. Eicosanoids production pathway

Eicosanoids (prostaglandins, thromboxanes and leukotrienes) are crucial players in inflammation which directly contribute to the symptoms of various diseases. The formation of arachidonic acid (AA) is catalyzed by a superfamily of PLA_2 enzymes, including secretory PLA_2 ($sPLA_2$), Ca^{2+}-independent PLA_2 ($iPLA_2$) and cytosolic PLA_2 ($cPLA_2$) [71]. Among this group, especially the group IVA $cPLA_2$ shows selectivity for AA-containing phospholipids and, therefore, represent critical enzymes in the regulation of eicosanoids synthesis [72]. Activation of $cPLA_2$ by Ca^{2+} and MAPK leads to its translocation from the cytosol to the plasma membrane, where it liberates AA from the phospholipids. The subsequent conversion of AA is mediated either by cyclooxygenases (COXs) to generate prostaglandins (PGs) and thromboxanes or by lipoxygenases to generate leukotrienes. Two COX isoforms exist, i.e. the constitutively expressed COX-1 and the inducible COX-2. Whereas COX-1 produces low levels of PGs for physiological purposes, COX-2 is considered to be induced under inflammatory conditions and to contribute to the high levels of

PGE_2 in inflammation. COXs catalyze the conversion of AA into the unstable intermediate metabolite PGG_2, which is subsequently reduced to PGH_2 – a precursor for different PGs (PGI_2, PGD_2, $PGF_{2\alpha}$, PGE_2 and thromboxane A_2). Once synthesized, PGH_2 is rapidly converted into prostanoids by the action of specific prostaglandin synthases. There are three particular synthases, which contribute to PGE_2 synthesis: microsomal PGE synthase-1 (mPGES-1), microsomal PGE synthase-2 (mPGES-2) and cytosolic PGE synthase (cPGES) [73]. mPGES-1 is frequently induced concomitantly with COX-2 by various inflammatory stimuli to generate a rapid increase of PGE_2 production, while mPGES-2 and cPGES are constitutively expressed and functionally coupled to COX-1 to maintain physiological levels of PGE_2 [74]. Induction of COX-2/mPGES-1 leads to the production of PGE_2, which uniquely signals via four specific GPCRs, named E prostanoid receptors (EP_{1-4}), and thus plays a major role in inflammation [75].

1.2.2. Implication of molecular players of inflammation in multiple disorders

The contribution of PGE_2 to inflammation has been demonstrated in many studies, all confirming the role of this lipid in inflammation-associated diseases. However, mPGES-$1^{-/-}$ mice are protected from a variety of inflammatory diseases, like collagen-induced arthritis and antigen-induced paw edema [76]. Based on these findings, targeting mPGES-1 has been considered as a possible therapeutic approach. mPGES1 is overexpressed in different types of cancers, including lung cancer and cervical cancer and its overexpression in experimental models promotes tumor growth, metastasis, angiogenesis and resistance to chemotherapy. Knockdown of mPGES-1 reduced PGE_2 synthesis, cell proliferation and invasion of Levis lung carcinoma cells *in vitro* and also reduced xenograft formation in mice *in vivo*, while its overexpression has the opposite effect [77].

Another enzyme essential for PGE_2 synthesis, COX-2, also contributes to cancer progression. Sustained induction of COX-2 expression promotes the transition from acute to chronic inflammation, which is a hallmark of carcinogenesis. Systemic inflammation facilitates tumor growth and metastasis by altering the tumor cell phenotype and activating stromal cells to generate a pro-tumorogenic microenvironment. Overexpression of COX-2 is found in many types of cancer, including lung, colon, breast, gastric and pancreas, and associated with poor prognosis and shorter overall survival [78–80]. There are several mechanisms underlying the carcinogenic activity of COX-2. Activation of COX-2/PGE_2 pathway induces proliferation of cancer cells [81], and also prevents apoptosis, thereby mediating resistance to chemotherapy [82]. Another mechanism is based on the pro-angiogenic activity of COX-2 [83]. Data from several studies suggest that expression of COX-2 in breast and lung cancer cells upregulates vascular endothelial growth factor C (VEGF-C), which, via VEGF receptor 3, promotes lymphoangiogenesis [84]. Furthermore, COX-2 expression can increase the migratory and invasive phenotype of cancer cells and is tightly linked to lymph node metastasis. Inhibition of COX-2 by the selective inhibitor celecoxib decreased tumor growth and metastasis in a xenograft model of breast cancer [85]. Involvement of the COX-2/mPGES-1/PGE_2 cascade was also demonstrated in models of kidney toxicity, such as an acute cisplatin-induced kidney injury in renal parenchymal cells [86]. Moreover, TGFβ triggered activation of the COX-2/mPGES-1 cascade, leads to increased formation of PGE_2, which, in turn, promotes proliferation of mouse mesangial cells via the EP_1 receptor [87].

An alternative target for the treatment of inflammatory disorders is the upstream located $cPLA_2$ as it catalyzes the rate-limiting step in the generation of PGE_2. In a chronic ear edema model in mice, an inhibitor of $cPLA_2$, $AACOCF_3$, exerted a suppressive effect on PMA-induced ear edema [88]. Furthermore, $cPLA_2$ inhibitors attenuated ischemia/reperfusion-induced lung

injury [89]. Additionally, in a mouse model of multiple sclerosis, the experimental autoimmune encephalomyelitis (EAE), which represents an inflammatory demyelinating disease, it was shown that the $cPLA_2$ inhibitor $AACOCF_3$ significantly reduces symptoms of EAE, indicating a role of PLA_2 in the pathogenesis of multiple sclerosis [90].

Taken together, these data suggest that PGE_2 synthesis is involved in numerous acute and chronic inflammation processes and that pharmacological interference in different steps of PGE_2 generation may be beneficial for treating inflammatory diseases.

Aim of the thesis

The aim of this thesis was to study the interconnection between the two lipid molecules S1P and PGE_2, and their contribution to proliferation and migration of non-cancerous cells associated with proliferative disorders (mesangial cells, embryonic fibroblasts), and of metastatic breast cancer cells. Another focus was to investigate the role of the bioactive sphingolipid molecule S1P in inflammation-associated diseases, and to identify the S1P receptor subtype involved in S1P signaling as a potential target to prevent these disorders. In addition, the mechanisms of regulation of inflammation were elucidated using pharmacological modulators targeting this signaling pathway.

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Chapter 2

Upregulation of the $S1P_3$ receptor in metastatic breast cancer cells increases migration and invasion by induction of prostaglandin E_2 and EP_2/EP_4 receptors activation

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Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids (2016) 1861 (11): 1840 – 51

All the results presented in this chapter (except of Fig. 5C,D,E and Fig. 5S, which were produced by Dr. Stephanie Schwalm) were produced by me under the supervision of Prof. Dr. Andrea Huwiler and Prof. Dr. Uwe Zangemeister-Wittke.

Abstract

Breast cancer is one of the most common and devastating malignancy among women worldwide. Recent evidence suggests that malignant progression is also driven by processes involving the sphingolipid molecule sphingosine 1-phoshate (S1P) and its binding to cognate receptor subtypes on the cell surface. To investigate the effect of this interaction on the metastatic phenotype, we used the breast cancer cell line MDA-MB-231 and the sublines 4175 and 1833 derived from lung and bone metastases in nude mice, respectively. In both metastatic cell lines expression of the $S1P_3$ receptor was strongly upregulated compared to the parental cells and correlated with higher S1P-induced intracellular calcium ($[Ca^{2+}]_i$), higher cyclooxygenase (COX)-2 and microsomal prostaglandin (PG) E_2 synthase expression, and consequently with increased PGE_2 synthesis. PGE_2 synthesis was decreased by antagonists and siRNA against $S1P_3$ and $S1P_2$. Moreover, in parental MDA-MB-231 cells overexpression of $S1P_3$ by cDNA transfection also increased PGE_2 synthesis, but only after treatment with the DNA methyltransferase inhibitor 5-aza-2-deoxycytidine, indicating reversible silencing of the COX-2 promoter. Functionally, the metastatic sublines showed enhanced migration and Matrigel invasion in adapted Boyden chamber assays, which further increased by S1P stimulation. This response was abrogated by either $S1P_3$ antagonism, COX-2 inhibition or prostaglandin E_2 receptor 2 (EP_2) and 4 (EP_4) antagonism, but not by $S1P_2$ antagonism. Our data demonstrate that in breast cancer cells overexpression of $S1P_3$ and its activation by S1P has pro-inflammatory and pro-metastatic potential by inducing COX-2 expression and PGE_2 signaling via EP_2 and EP_4.

2.1. Introduction

Breast cancer is the most common and devastating malignancy among women behind lung cancer and its metastasis is a major cause of poor prognosis [1,2]. The proliferative, migratory and invasive properties of metastatic cancer cells undermine the efficacy of available therapies, but more effective treatments for advanced breast cancer can only be developed if the molecular mechanisms are better understood [3,4]. Recent evidence suggests that inflammation is often involved in the malignant progression of solid tumors, including breast cancer, and chronic inflammation is a major risk factor for breast cancer development, treatment resistance and recurrence [5,6]

Although the link between inflammation and cancer initiation is well established by its ability to generate a pro-proliferative and pro-angiogenic environment, its role in metastatic diseases, has been poorly explored. Prominent mediators of cancer inflammation include the oncogenic NFκB and several cytokines like IL-6, IL-8 and IL-1β, which are activated and overexpressed in breast cancer [7,8]. In addition, biologically active lipids like eicosanoids are implicated in inflammation and cancer too. The cyclooxygenase isoform-2 (COX-2) is frequently overexpressed in solid tumors and activated by different cytokines and growth factors to support inflammatory processes and migration [9,10]. It generates the metabolite prostaglandin E_2 (PGE_2), which promotes cancer cell growth, stimulates angiogenesis, migration and invasion, confers resistance to apoptosis and suppresses immunity [11,12]. Also in breast cancer, elevated expression of COX-2 and overproduction of downstream PGE_2 is associated with a metastatic phenotype and poor prognosis [13,14]. The biological effect of PGE_2 is mediated by binding to EP receptors of which four subtypes have been characterized [15,16]. Particularly, EP_2 and EP_4 have been found to be closely associated with an invasive phenotype in various cancer types [17,18]. In breast cancer cells COX-2 induced PGE_2 synthesis was shown

to increase expression of the pro-invasive CCR7 chemokine via EP_2 and EP_4 activation, and in breast cancer tissues overexpression of COX-2 correlates with lymph node metastasis [14].

Recent findings corroborate that malignant progression and aggressiveness of breast cancer is also driven by processes involving the bioactive sphingolipid metabolite sphingosine 1-phosphate (S1P). S1P has a pro-inflammatory potential [19,20] and stimulates cancer-relevant responses like cell growth, migration, invasion and angiogenesis [21] . Although S1P shows also intracellular distribution, most of its biological effects result from binding to cognate G protein-coupled receptors (GPCRs) on the cell surface [22,23]. Five subtypes of S1P receptors ($S1P_{1-5}$), have been identified, each of it demonstrates tissue-specific expression and couples to different G proteins [24]. As shown in renal mesangial cells and smooth muscle cells, S1P binding to $S1P_2$ and $S1P_3$ can induce COX-2 expression and PGE_2 synthesis [25,26], and from lung cancer cells there is evidence that it activates $cPLA_2$ to produce arachidonic acid via $S1P_3$ [27]. In line with these findings, S1P was also reported to induce PGE_2 synthesis through activation of $S1P_1$ and $S1P_3$ in non-tumorigenic granulosa cells [28]. Furthermore, increased formation of S1P with subsequent activation of $S1P_1$ and the STAT3 pathway links chronic intestinal inflammation to colitis-associated carcinogenesis [29], and in breast cancer S1P was shown to induce migration and invasion via $S1P_3$-mediated upregulation of MMP-9 [30]. S1P is also able to transactivate tyrosine kinase receptors such as the EGF receptor and thereby increase EGFR signaling in breast cancer cells [31]. On the other hand, in non-malignant cell types anti-inflammatory effects of S1P mediated by $S1P_1$, $S1P_2$ and/or $S1P_3$ have been described [32,33], a discrepancy which needs to be clarified by further investigations.

In the present study, we used the breast cancer cell line MDA-MB-231 and two sublines established from single cell-derived progenies derived from lung and bone metastases in nude mice [34,35] to investigate S1P-induced pro-

inflammatory effects associated with their increased metastatic potential. We report that in both metastatic sublines $S1P_3$ is overexpressed and its activation by extracellular S1P stimulates COX-2 expression and PGE_2 synthesis. PGE_2 binding to EP_2 and also EP_4 on the metastatic cells increases migration and Matrigel invasion in an auto- and paracrine manner thus underpinning their aggressive phenotype.

2.2. Materials and methods

2.2.1. Chemicals and reagents

The PGE_2-specific ELISA was obtained from Enzo Life Science (Lörrach, Germany); human recombinant IL-1β was from PeproTech (Hamburg, Germany). Secondary HRP-coupled IgGs, hyperfilm MPR and enhanced chemiluminescence reagents were purchased from GE Health Care Systems (Glattbrugg, Switzerland). Antibody against COX-2 was from Cell Signaling (Schwalbach, Germany); antibody against microsomal prostaglandin E synthase-1 (mPGES-1) was from Cayman Chemicals Inc. (Ann Arbor, MI, US); antibody against β-actin (clone AC-15) was from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland); S1P, VPC23019 and W146 were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA); JTE013, TY52156, SC51322, PF04418948, L-798.106, were from Tocris Bioscience (Bristol, United Kingdom); AH23848, digitonin, celecoxib, CYM5541, PGE_2, 5‘-aza-2‘-deoxycitidine (aza-C) and KICqStart®SYBR®Green qPCR ReadyMix™ (SYBRgreen) were obtained from Sigma Aldrich Chemie GmbH (Buchs, Switzerland); fura-2/AM was from LuBio Science GmbH (Luzern, Switzerland); TurboFect™ was from Fermentas Life Science/ThermoFisher Scientific (Zug, Switzerland); First Strand DNA Synthesis Kit, was from MBI

Fermentas (St-Leon-Roth, Germany); TRIZOL® reagent (5-Prime), Corning® Matrigel® Basement Membrane Matrix and growth factor reduced (GFR) (Matrigel) were from VWR International AG (Dietikon, Switzerland); all cell culture media and supplements were purchased from Invitrogen AG (Basel, Switzerland); PCR primers were purchased from Sigma-Aldrich Chemie GmbH.

2.2.2. Cell lines and cell culture conditions

The human breast cancer cell line MDA-MB-231 and its sublines 4175 and 1833, which were derived by single-cell cloning from lung and bone metastases of nude mice, respectively [34], are from Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 10 mM HEPES pH 7.4, 100 units / ml penicillin and 100 µg/ml streptomycin. All cells were grown at 37^0 C in a humidified atmosphere containing 5 % CO_2.

For $S1P_2$ and $S1P_3$ downregulation, cells were transfected with Oligofectamine and 100 nM of a Smartpool siRNA of $hS1P_2$ (Dharmacon, ON-TARGETplus SMARTpool S1PR2 (L-003952) or $hS1P_3$ (Dharmacon, ON-TARGETplus SMARTpool S1PR3 (L-005208)) as recommended by the manufacturer in 24-well-plates. Control siRNA transfection was performed with non-targeting siRNA (On-TARGETplus Control D-001810). 48 h after transfection cells were incubated under serum free conditions for 24 h and then stimulated with S1P or vehicle for 2 h.

2.2.3. $S1P_3$ overexpression in MDA-MB-231 cells

The human $S1P_3$ pcDNA3.1 construct was kindly provided by Dr. D. Guerini (Novartis, Basel). Transfection was performed using TurboFect™ following the manufacturer's instructions. Briefly, parental MDA-MB-231 cells were subcultured into a 6-well plate for 24 hours to achieve ~70% confluency

on the day of transfection. 2 µg of DNA and 6 µl of transfection reagent were mixed in 200 µl serum free DMEM and placed drop-wise to each well for 48 hours. Stable transfectants were obtained under selection conditions in the presence of 0,5 mg/ml G418 for 10 -15 days.

2.2.4. Quantitative real-time PCR

Total RNA (1 µg) isolated with TRIZOL® reagent (5-Prime) was used for reverse transcriptase PCR using the First Strand DNA Synthesis Kit and a random hexamer primer for amplification. The real-time PCR reaction was performed using SYBRgreen and a BioRad iQ5 Cycler Detection System (BioRad Laboratories AG, Glattbrugg, Switzerland). The primer sequences used for qPCR are listed in Table 2.1. The fold induction values were obtained according to the $\Delta\Delta C_T$ method after normalization to the housekeeping gene 18S RNA.

Table 2.1. Characteristic of primers used for qPCR analysis

Gene	Forward primer (from 5' to 3') Reverse primer (from 5' to 3')
human $S1P_1$	CCTCTAGCGTTCGTCTGG TTTCCCGTGTAGTTGTAATGC
human $S1P_2$	TCAAGACGGTCACCATCG ACAGGCATAGTCCAGAAGG
human $S1P_3$	CTTACGACGCCAACAAGAG CAGGATGGTAGAGCAGTCAG
human $S1P_4$	TGTTCTGCACTACAACCACTC AAGTTCTCCAGCACCACCAG
human $S1P_5$	TCTTCCTGCTGCTGTTGCTC GCGTGTAGATGATGGGGTTCAG
human COX-1	CTAAAGATTGCCCCACACC GTTTCCCATCCTTAAAGAGCC

Gene	Forward primer (from 5' to 3') Reverse primer (from 5' to 3')
human COX-2	TGACAGTCCACCAACTTACAATGC AATCATCAGGCACAGGAGGAAGG
human EP_1	CACGTGGTGCTTCATCGGCCTGGGTC CACCACCATGATACCGACAAG
human EP_2	TCCTGTTCTGAGACTAATGCGTTC GTTTACRGGCATCTGACTGTGTAG
human EP_3	GGGCCTGATGGAAGGTGTTT TCTATCATGAGAACTGCACCAAGTC
human EP_4	TCTGACCTCGGTGTCCAAAAATCG TGGGTACTGCAGCCGCGAGCTA

2.2.5. Cell homogenization and Western blotting

Cells were washed with phosphate-buffered saline (PBS), scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X100, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 1 µM pepstatin A, 1 mM phenylmethyl sulphonyl fluoride) and homogenized by passing 10 times through a 26-G needle fitted to a 1 ml syringe. Samples were centrifuged for 10 min at 13'000 x g. The supernatant was taken for protein determination and 30 µg protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane and subjected to Western blotting as described [36] using the antibodies indicated in the figure legends.

2.2.6. Measurement of $[Ca^{2+}]_i$ in cell suspension

Intracellular Ca^{2+} was measured essentially as described [37]. Briefly, after starvation for 24 hours, cells were washed and suspended in HBSS (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose and 15 mM Hepes (pH 7.4), and loaded for 45 min with 1 µM of the Ca^{2+} indicator dye fura-2/AM. Then, the cells were washed twice in HBSS and fura-2 fluorescence

was monitored in a spectrofluorometer (Hitachi) using excitation wavelengths of 340/380 nm and an emission wavelength of 510 nm. At a stable baseline (250 sec), S1P was added to a final concentration of 1 µM and the change of fluorescence was recorded. At the end of each scan (550 sec), 10 µM of digitonin was added to determine the maximum Ca^{2+} and 10 mM of the chelating agent EDTA was added (750 sec) to achieve the minimum concentration of Ca^{2+} in the cell. Data were calculated using the FL WinLab program (PerkinElmer AG, Schwerzenbach, Switzerland).

2.2.7. Measurement of secreted prostaglandin E_2

PGE_2 in cell culture supernatants was quantified using a competition immunoassay (PGE_2-ELISA/EIA kit from Enzo Life Sciences, Lörrach, Germany). 100 µl cell supernatants were processed according to the manufacturer's instructions. The absorbance at 405 nm was measured by spectrophotometry and the PGE_2 concentration was determined with a calibration curve using PGE_2 as standard.

2.2.8. Migration and invasion assays

Cell migration and invasion were measured with an adapted Boyden chamber assay. For invasion measurement, transwell filters had been precoated with 100 µl of 6 mg/ml Matrigel. Quiescent cells at a density of 2,5 x 10^5 cells in 200 µl starvation medium (0.1mg/ml^{-1} fatty acid-free bovine serum albumin) were seeded into the transwell filters (6,5 µm diameter, 8 µm pore size). 750 µl of serum free or complete growth medium (for migration and invasion assays, respectively) was placed in the lower compartment and cells were incubated at 37°C for the indicated time periods to allow migration/invasion. Thereafter, transwell filters were removed and cells which had migrated/invaded through the filter into the lower chamber were counted in four different random fields

for one sample using a Nikon TMS-F microscope (Nikon AG, Egg/ZH, Switzerland).

2.2.9. Statistical analysis

Statistical analysis of data was performed using one-way ANOVA following Dunnett post-hoc test for multiple comparisons or unpaired t-test when only two groups were compared.

2.3. Results

2.3.1. $S1P_3$ is upregulated in metastatic breast cancer cells and its activation by S1P induces intracellular calcium mobilization

The role of S1P and its cognate cell surface receptors in breast cancer metastases is still unclear. Here, we used the breast cancer cell line MDA-MB-231 and the two sublines 4175 and 1833 derived from lung and bone metastases, respectively, in nude mice [34], to determine the S1P receptor subtype associated with the metastatic phenotype. First, parental and metastatic MDA-MB-231 cells were characterised for S1P receptor expression using quantitative PCR. As shown in Fig. 1, the parental MDA-MB-231 cells showed the highest mRNA levels for $S1P_3$ and $S1P_5$ among the different receptor subtypes, low expression of $S1P_1$ and $S1P_2$, whereas $S1P_4$ was undetectable. For comparison, in both metastatic sublines expression of $S1P_3$ was strongly enhanced and reached particularly high levels in 1833 cells. The $S1P_1$ was downregulated in both metastatic sublines whereas the other S1P receptors showed a similar expression pattern as in parental MDA-MB-231 cells (Fig. 1).

To verify that overexpression of $S1P_3$ in the metastatic MDA-MB-231 sublines is coupled to increased $S1P_3$ receptor signalling, we investigated whether Ca^{2+} mobilization as an early read-out of receptor activation [23,37]

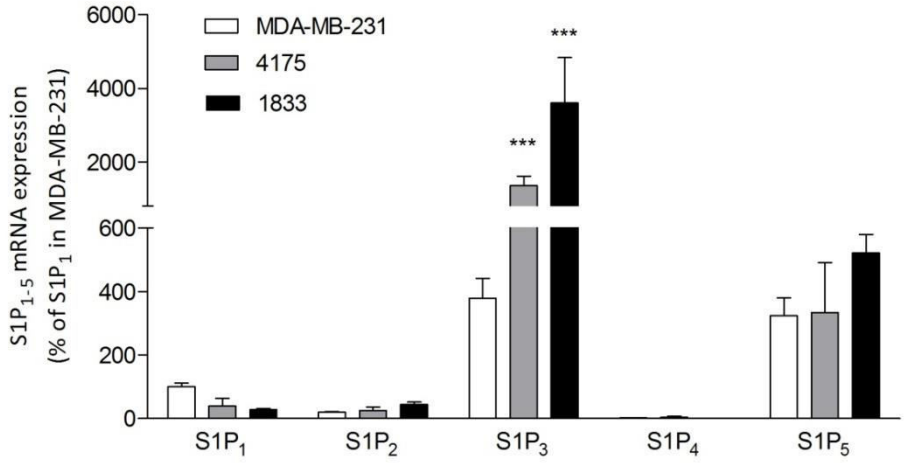


Fig. 1. mRNA expression profile of $S1P_{1-5}$ receptors in parental and metastatic MDA-MB-231 cells. Confluent cells were incubated in serum-free medium for 24 h. Thereafter, RNA was extracted and taken for quantitative PCR analysis of $S1P_{1-5}$ using the primers indicated in the Materials and Methods section. 18S RNA was used for normalization. Data are expressed as a % to the $S1P_1$ expression in parental MDA-MB-231 cells and are the mean ± SD (n=4). $^{***}p< 0.0001$ compared to $S1P_1$ expression in parental MDA-MB-231 cells.

increased in the presence of extracellular S1P. To this end, cells were loaded with the Ca^{2+} indicator fura-2/AM and stimulated with S1P. As shown in Fig. 2A, the parental MDA-MB-231 cells responded to S1P with only a marginal increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$). In contrast, in both metastatic sublines, which had higher basal ($[Ca^{2+}]_i$ levels, S1P induced a 6- to 8-fold increase of $[Ca^{2+}]_i$ compared to the parental cells (Fig. 2A, B). In all cell lines, pre-treatment with the $S1P_{1/3}$ antagonist VPC23019 effectively blocked S1P-induced Ca^{2+} mobilization. The $S1P_1$ antagonist W146 had no effect on any of the cell lines and the $S1P_2$-selective antagonist JTE013 suppressed $[Ca^{2+}]_i$ increase only in the parental cells (Fig. S1A, S1B). A selective $S1P_5$ antagonist is not yet available and therefore, this receptor subtype could not be explicitly excluded. The increase in baseline Ca^{2+} mobilization measured upon pre-treatment of 4175 cells with all three antagonists (Fig. S1A) might be due to a decreased inhibitory

activity of the G_i-coupled receptors. The pattern of responsiveness to the various antagonists together with the receptor subtype expression profiles shown in Fig. 1 confirms that $S1P_3$ overexpressed on the metastatic MDA-MB-231 cells is fully functional and represents the major receptor involved in S1P-induced Ca^{2+} mobilization.

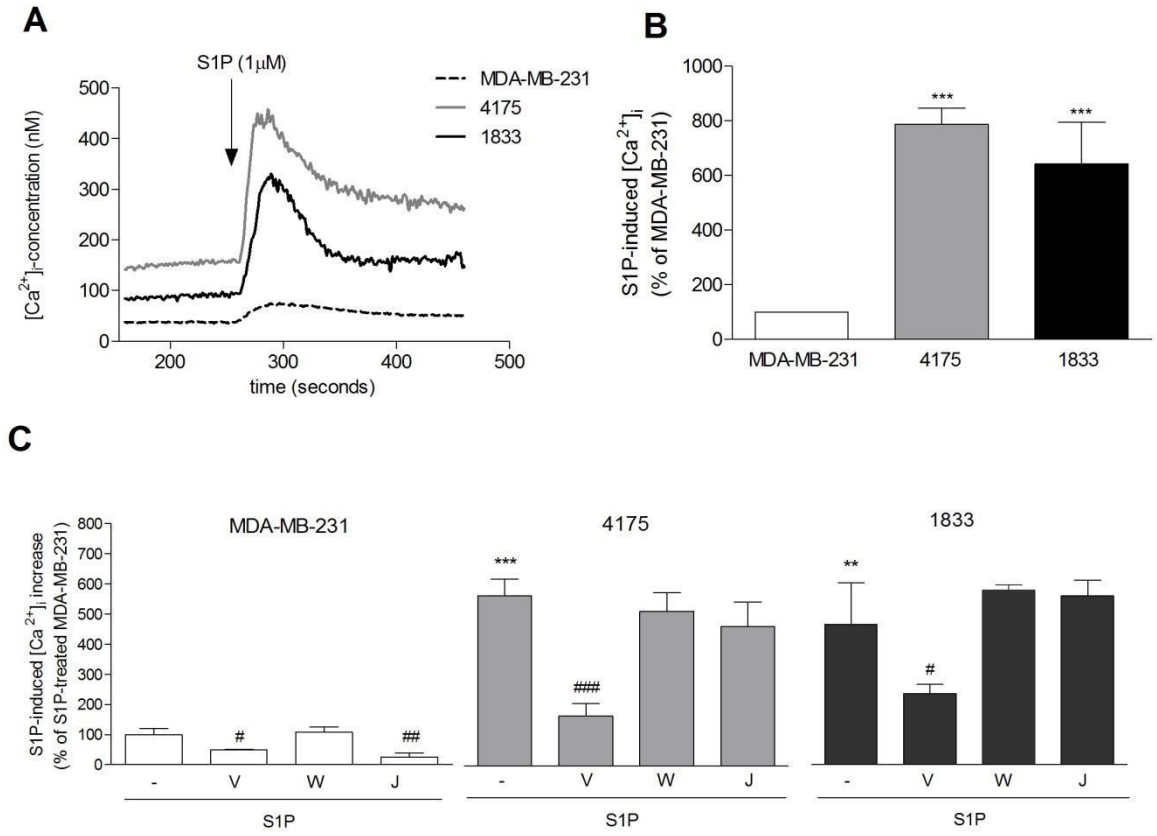


Fig. 2. Effect of exogenous S1P on intracellular calcium $[Ca^{2+}]_i$ mobilization in parental and metastatic MDA-MB-231 cells. Cells were loaded with fura-2/AM prior to stimulation with 1 μM S1P and $[Ca^{2+}]_i$ was measured as described in the Materials and Methods section. (A) Representative curves from parental MDA-MB-231 cells and the metastatic sublines. (B) Maximum S1P-induced peak level of Ca^{2+} expressed as % of the parental MDA-MB-231 cells. Data are the mean ± SD (n=3). $^{***}p< 0.001$ compared to parental MDA-MB-231 cells. (C) Cells were pretreated for 10 min with the $S1P_{1/3}$ antagonist VPC23019 (V, 10 μM), the $S1P_1$ antagonist W146 (W, 10 μM) or the $S1P_2$ antagonist JTE013 (J, 10 μM) prior to stimulation with 1 μM S1P. $[Ca^{2+}]_i$ was measured and peak levels determined. Data are the mean ± SD (n=3). $^{**}p<0.01$, $^{***}p<0.001$, compared to parental MDA-MB-231 cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to the corresponding S1P-treated cells.

2.3.2. COX-2 and mPGES-1 are upregulated in metastatic breast cancer

There is evidence that COX-2 and its product PGE_2 are implicated in breast cancer metastasis [13,14]. Here, we investigated whether COX-2 and PGE_2 are also upregulated in the two metastatic MDA-MB-231 sublines. As shown in Fig. 3 A, the untreated metastatic sublines already produce higher basal amounts of PGE_2 compared to the parental MDA-MB-231 cells. Treatment of cells with the inflammatory cytokine interleukin (IL)-1β together with forskolin (Fk), a regimen known to synergistically upregulate PGE_2 [38], only very slightly increased PGE_2 in the parental cells but triggered a strong increase in both metastatic sublines (Fig. 3A). As shown in Fig. 3B and Fig. S2, basal COX-2 and COX-1 mRNA expression was higher in the metastatic cells compared to the parental cells. Most importantly, however, similar to PGE_2 synthesis IL-1β/Fk strongly increased COX-2 mRNA expression (Fig. 3B) and protein expression (Fig. 3D) in the metastatic cells, whereas COX-1 expression even slightly decreased (Fig. S2). This strongly suggests that the high level of PGE_2 was produced by COX-2. Again, in the parental MDA-MB-231 cells the effect of IL-1β/Fk on COX-2 and COX-1 expression was marginal (Fig. 3B, Fig. S2).

Another downstream enzyme which is involved in PGE_2 synthesis and inducible by inflammatory mediators is the microsomal prostaglandin E synthase-1 (mPGES-1). We found that in the metastatic MDA-MB-231 cells mPGES-1 is also upregulated on the mRNA and protein level, and is strongly inducible by IL-1β/Fk (Fig. 3C, D). Again, in the parental MDA-MB-231 cells the effect of IL-1β/Fk on mPGES-1 expression was low (Fig. 3C, D). Since IL-1β/Fk is known as a strong inducer of COX-2 and mPGES-1 in many cell types, we wondered whether the here observed weak effect in the parental MDA-MB-231 cells was due to suppression by promoter silencing, as such epigenetic regulation was reported also for other genes during metastatic selection in this cell line [39]. In fact, treatment of the parental cells with the DNA

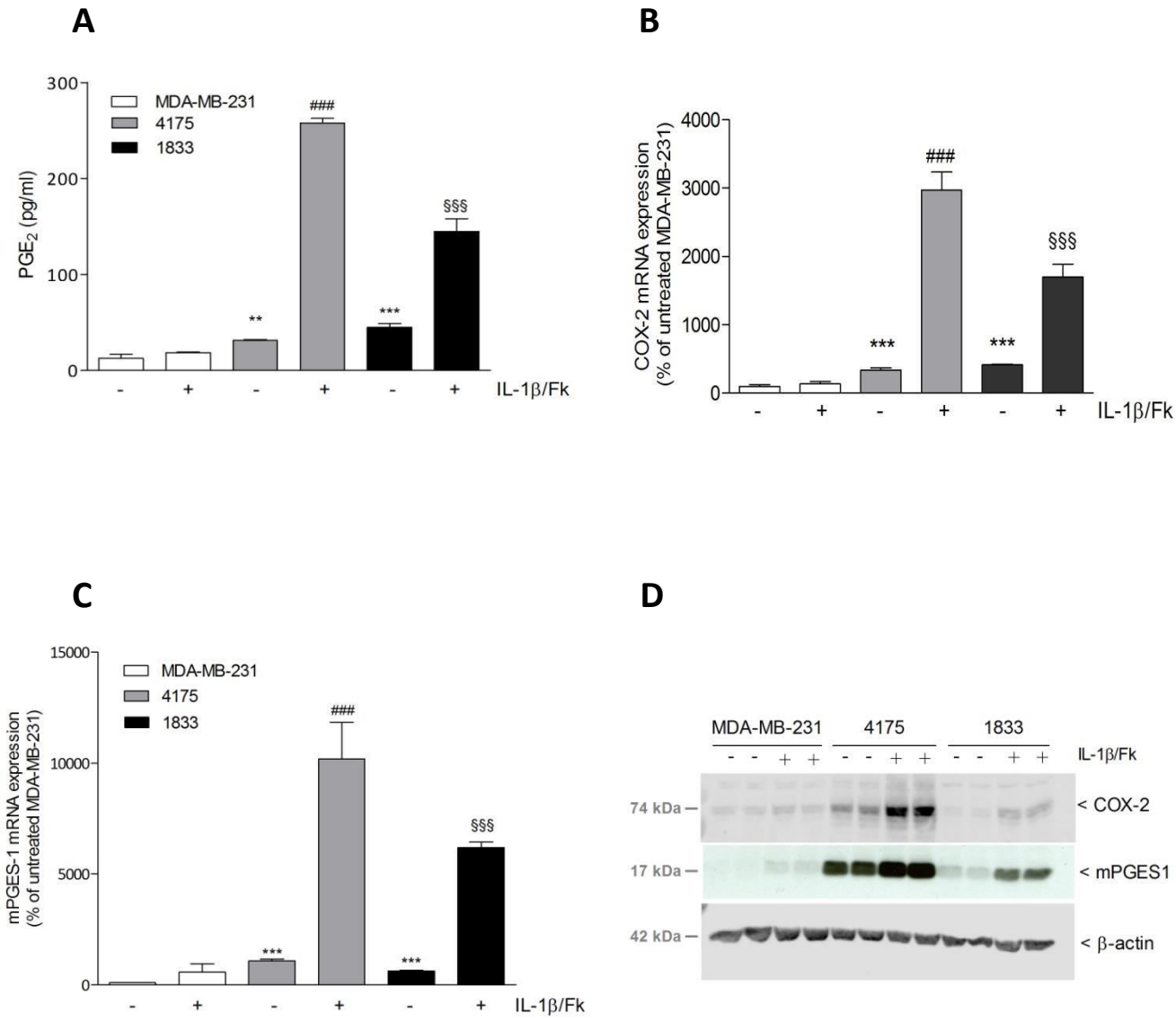


Fig. 3. PGE_2 synthesis and mRNA expression of COX and mPGES-1 in parental and metastatic MDA-MB-231 cells. Cells were incubated for 24 h in serum-free medium prior to stimulation for 24 h with either vehicle control (-) or a combination of IL-1β (1 nM) plus forskolin (Fk, 5 μM). (A) Supernatants were taken for PGE_2 quantification by ELISA as described in the Materials and Methods section. Data are expressed as pg of PGE_2 per ml supernatant and are the mean ± S.D. (n=3). (B and C) Cells were taken for RNA extraction and quantitative PCR analysis of COX-2 (B) and mPGES1 (C). Data are the mean ± S.D. (n=3). **p<0.01, ***p<0.001 compared to unstimulated parental MDA-MB-231 cells; $^{###}$p<0.001 compared to unstimulated 4175 cells; §§§p < 0.001, compared to unstimulated 1833 cells. (D) Cells were taken for protein extraction, separation by SDS-PAGE (8 % gels for COX-2 and β-actin, 15 % gels for mPGES-1) and Western blotting using antibodies against COX-2 (upper panel), mPGES-1 (middle panel) and β-actin (lower panel). Blots are representative of three independent experiments showing similar results.

methyltransferase inhibitor 5-aza-2'-deoxycitidine (aza-C) increased COX-2 mRNA and protein expression (Fig. S3A, B), suggesting that the COX-2 promoter is silenced in these cells by methylation.

2.3.3. S1P induces COX-2 expression and PGE_2 synthesis in metastatic breast cancer cells via $S1P_3$

To investigate the possibility that the high COX-2 expression and PGE_2 synthesis in the metastatic MDA-MB-231 cells is induced by S1P via overexpressed $S1P_3$, we treated parental and metastatic cells with exogenous S1P and measured COX-2 mRNA expression and PGE_2 synthesis at different time points. Fig. 4A shows that in parental MDA-MB-231 cells S1P induced only a marginal time-dependent increase of COX-2 mRNA expression, consistent with the finding that the COX-2 gene is silenced in these cells. On the other hand, in the two metastatic sublines S1P induced a strong time-dependent increase in COX-2 mRNA expression with peak values reached after 2 to 4 hours, which then returned to low basal levels. COX-2 protein expression behaved similarly, peaked at 2-4 hours and thereafter declined to control levels over the next hours (data not shown). As expected, quantification of PGE_2 in the cell culture supernatants revealed that S1P strongly accelerated PGE_2 synthesis in the metastatic cells compared to the parental cells (Fig. 4B). Notably, the secreted PGE_2 levels remained constantly high over 24 hours of S1P stimulation. The basal PGE_2 secretion over time was low but steadily increased over 24 h (Fig. S4)

To further investigate the mechanism underlying S1P-induced PGE_2 overproduction, the COX-2-selective inhibitor celecoxib was used. As shown in Fig. 5A, celecoxib dose-dependently blocked S1P-stimulated PGE_2 synthesis in the metastatic cells, confirming that particularly the COX-2 enzyme is responsible for PGE_2 overproduction induced by S1P. Furthermore, the use of various S1PR antagonists demonstrated that S1P-induced PGE_2 synthesis is

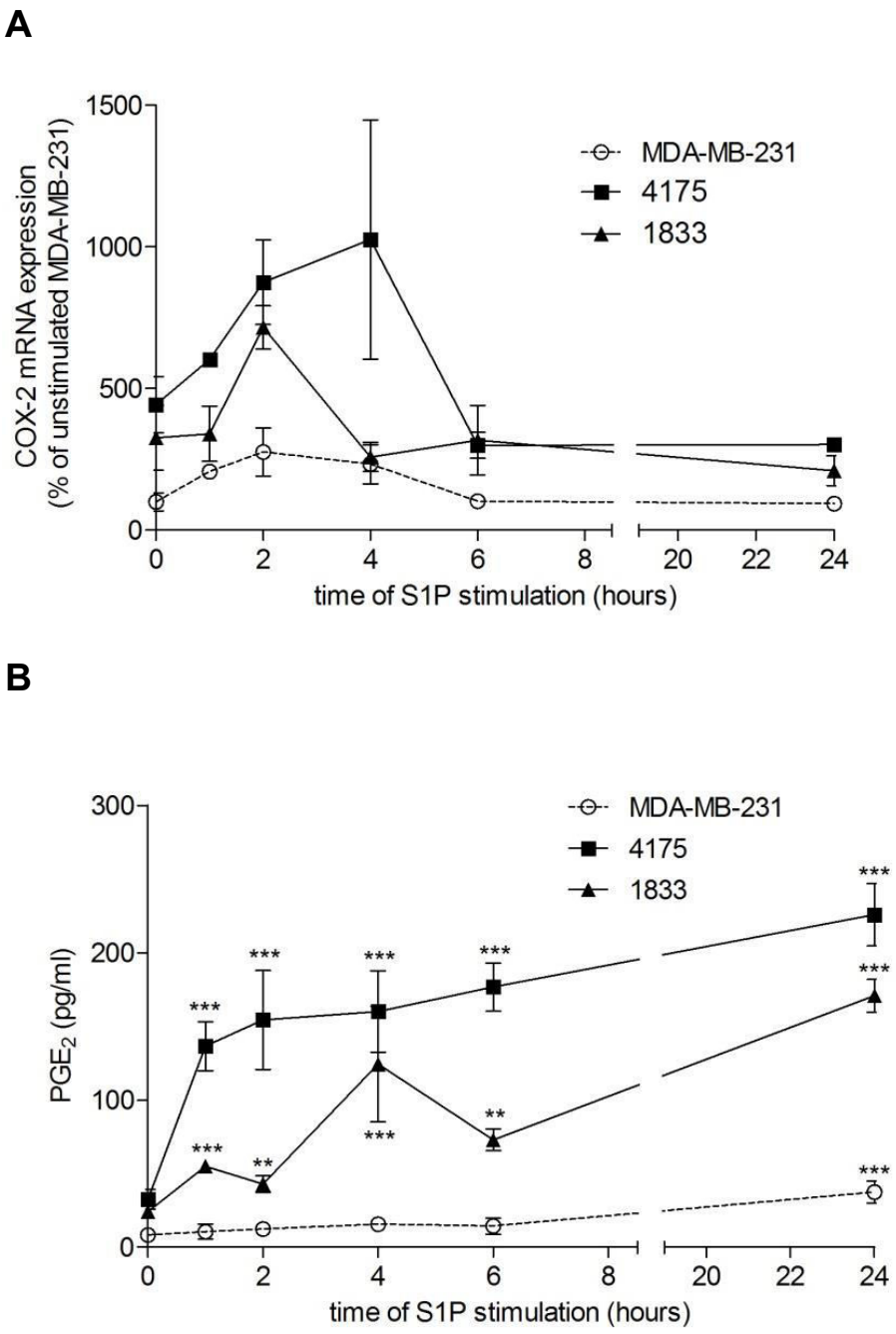
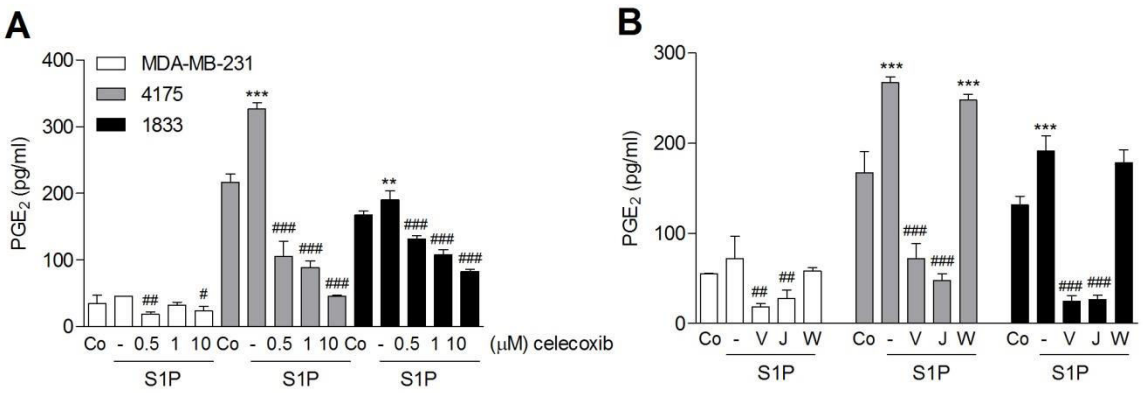


Fig. 4. Time-dependent effect of S1P on COX-2 mRNA expression and PGE_2 formation in parental and metastatic MDA-MB-231 cells. Cells were stimulated for 1-24 h with S1P (1 µM). (A) Cells were taken for RNA extraction and subjected to quantitative PCR analysis of COX-2. 18S RNA expression levels were used for normalization. Data are the mean (n=2). (B) Cell supernatants were collected for PGE_2 determination by ELISA. Data are expressed as pg of PGE_2 per ml of supernatant and are the mean ± S.D. (n=3). **p<0.01, ***p<0.001 compared to the corresponding unstimulated control cells.

effectively abolished by the $S1P_{1/3}$ antagonist VPC23019 and the $S1P_2$ antagonist JTE013, but not by the $S1P_1$-selective antagonist W146, suggesting that $S1P_2$, despite its low expression, and $S1P_3$ are involved (Fig. 5B). The selective $S1P_3$ agonist CYM5541 and the selective $S1P_2$ agonist CYM5520 also enhanced PGE_2 synthesis (Fig. 5C), thus strongly suggesting a critical role of both receptors. In a different approach, we downregulated $S1P_2$ and $S1P_3$ in 4175 cells by transfection with specific siRNAs. This downregulated approx. 80% of $S1P_2$ and 85-90% of $S1P_3$ in lung and bone metastatic cells (Fig. S5) and both treatments also inhibited S1P-stimulated PGE_2 synthesis to a similar extent (Fig. 5D), thus confirming that both receptors are involved in PGE_2 synthesis.

In addition, to confirm that $S1P_3$ activates PGE_2 synthesis, we stably overexpressed $S1P_3$ in parental MDA-MB-231 cells (Fig. 6A). Consistent with the fact that the COX-2 promoter is silenced in these cells, overexpression of $S1P_3$ alone and upon stimulation with S1P did not increase PGE_2 production (Fig. 6B). However, when the $S1P_3$-overexpressing cells were stimulated with S1P in the presence of the demethylating agent aza-C, PGE_2 production was strongly increased compared to untransfected control cells (Fig. 6B).



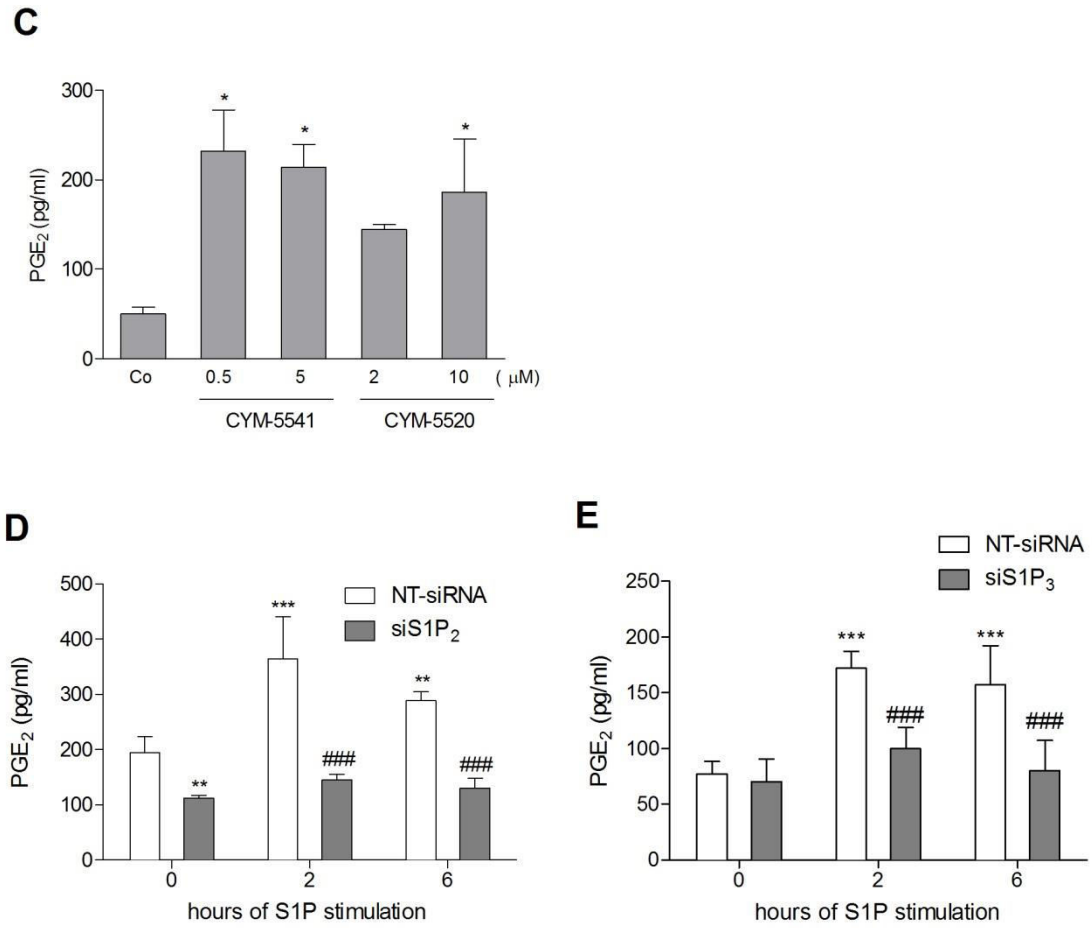


Fig. 5. Effect of celecoxib and S1PR modulators on S1P-induced PGE_2 synthesis in parental and metastatic MDA-MB-231 cells. (A) Cells were stimulated for 24 h with either vehicle control (Co) or 1 μM S1P in the absence (-) or presence of the indicated concentrations of celecoxib. (B) Cells were stimulated for 24 h with either vehicle (Co) or S1P (1 μM) in the absence (-) or presence of the $S1P_{1+3}$ antagonist VPC23019 (V, 10 μM), the $S1P_2$ antagonist JTE013 (J, 10 μM), the $S1P_1$ antagonist W146 (W, 10 μM) or with the $S1P_3$ agonist CYM5541 (C, 10 μM). All antagonists were added 30 min prior to S1P stimulation. (C) 4175 cells were stimulated for 2 h with the $S1P_3$ agonist CYM5541 (0.5 and 5 $^{-}$M) or the $S1P_2$ agonist CYM5520 (2 and 10 μM). (D and E) 4175 cells were transfected with either control siRNA (D and E, NT-siRNA), siRNA against $S1P_2$ (D, siS1P2) or siRNA against $S1P_3$ (E, siS1P3) as described in the Methods section. Thereafter, cells were stimulated for 2 h and 6 h with S1P (1 $^{-}$M). Supernatants were taken for PGE_2 quantification by ELISA. Data are the mean ± S.D. (n=3). **p<0.01, ***p<0.001 compared to the corresponding unstimulated vehicle-treated cells; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 compared to the corresponding S1P-stimulated cells.

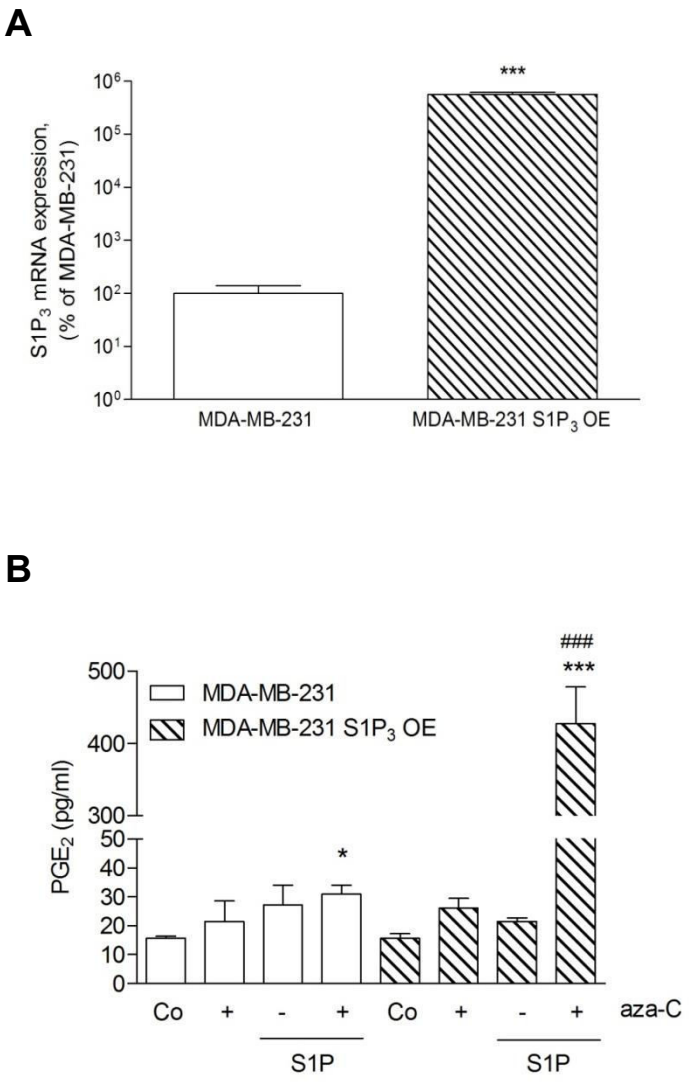


Fig. 6. Effect of S1P$_3$ overexpression in parental MDA-MB-231 cells on PGE$_2$ synthesis. (A) Parental MDA-MB-231 cells were either treated with vehicle control or transfected with a pcDNA3.1 vector containing the human S1P$_3$ cDNA (OE) for 48 h. Thereafter, cells were taken for RNA extraction and quantitative PCR analysis of S1P$_3$. Data are the mean ± S.D. (n=3). (B) Untransfected MDA-MB-231 cells and S1P$_3$ overexpressing cells (OE) were treated with vehicle control (Co) or 5 µM aza-C (+) for 3 days (fresh aza-C was added every 24 h). Then, cells were starved for 4 h and subsequently stimulated for 2 h with S1P (1 µM). Supernatants were taken for PGE$_2$ quantification by ELISA. Data are the mean ± S.D. (n=3). *p<0.05, ***p<0.001 compared to the corresponding vehicle-treated untransfected control cells; $^{###}$p<0.001 compared to the corresponding aza-C-treated cells.

2.3.4. S1P/$S1P_3$ increases migration of metastatic breast cancer cells in a COX-2 and PGE2-dependent manner

PGE_2 is a major pro-migratory prostanoid and implicated in metastasis [12]. Here, we investigated whether the high expression level of $S1P_3$ contributes to the metastatic phenotype of the MDA-MB-231 sublines by mediating S1P-induced COX-2 and PGE_2 synthesis. To this end, the migratory potential of cells was determined using an adapted Boyden chamber assay. As expected, the metastatic sublines migrated significantly faster than the parental cells and also strongly responded to S1P (Fig. 7A). Similarly, PGE_2 added to the upper chamber further strongly enhanced the migration of the metastatic variants whereas the parental cells were only marginally responsive (Fig. 7A). As shown in Fig. 7B, the $S1P_{1/3}$ antagonist VPC23019 and the recently developed $S1P_3$-selective antagonist TY52156 [40] both effectively offset S1P-induced migration. In contrast, the $S1P_2$ antagonist JTE-013 failed to reduce S1P-stimulated migration (Fig. 7C).

Next, we examined, whether COX-2 is directly involved in the pro-migratory effect of S1P. To this end, we treated cells with the selective COX-2 inhibitor celecoxib to abrogate PGE_2 synthesis. Fig. 7D shows that celecoxib indeed inhibited S1P-induced cell migration in both metastatic sublines. This suggests that the pro-migratory effect of S1P/$S1P_3$ signaling is mediated by increased PGE_2 synthesis and secretion, followed by activation of cognate EP receptors on the metastatic cells in an auto- and paracrine manner.

In a control experiment presented in Fig. 7E, we examined the pro-migratory effect of S1P on parental cells MDA-MB-231 cells overexpressing $S1P_3$ by transfection. In untransfected control cells S1P alone induced a measurable but marginal increase of migration as expected from the relative lack of COX-2 and PGE_2 expression in these cells. Surprisingly, however, we found that also aza-C had only a very marginal stimulatory effect and even in

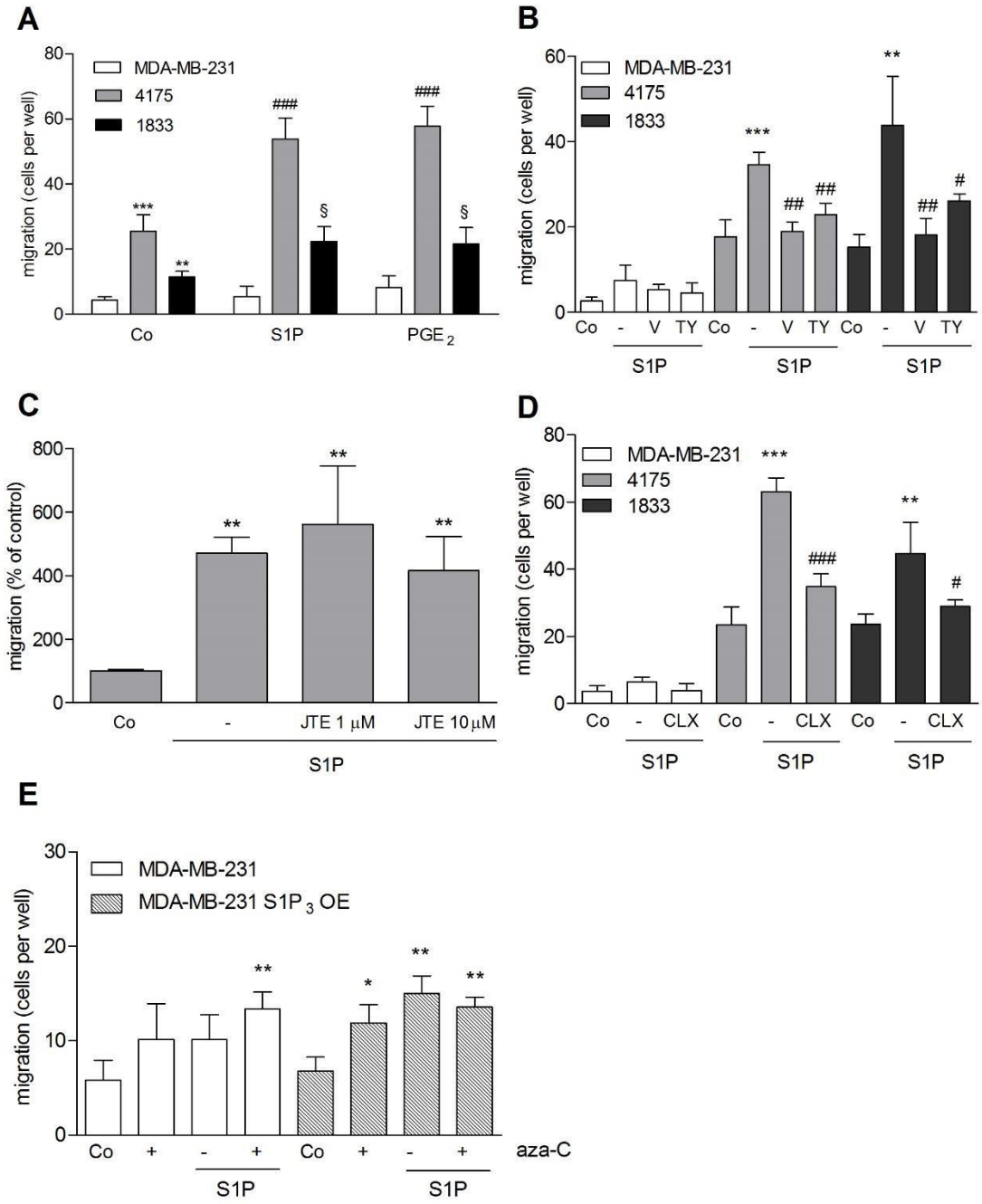


Fig. 8. Effect of S1P and PGE_2 on the migration of parental $S1P_3$-overexpressing MDA-MB-231 cells and of metastatic MDA-MB-231 cells. (A) 2 x 10^5 parental and metastatic cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co), S1P (1 μM) or PGE_2 (1 μM) under serum-free conditions. (B) 2 x 10^5 parental and metastatic cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 μM) in the absence (-) or presence of the $S1P_3$ antagonists VPC23019 (V, 10 μM) or TY52156 (TY, 1 μM). (C) 4175 cells were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 μM) in the absence (-) or presence of the $S1P_2$ antagonist JTE013 (1 μM and 10 μM). (D) 2 x 10^5 parental and metastatic cells

were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 µM) in the absence (-) or presence of celecoxib (CLX, 5 µM). (E) 2 x 10^5 parental and $S1P_3$-overexpressing cells (OE) were seeded onto transwell filters and treated for 20 h with either vehicle (Co) or S1P (1 µM) in the absence or presence of aza-C. Migrated cells were determined as described in the Materials and Methods section. Data are expressed as number of cells migrated to the lower chamber cells and are the mean ± SD (n=3). In A: **p<0.01, ***p<0.001 compared to vehicle-treated parental MDA-MB-231 cells; $^{###}$p<0.001 compared to vehicle-treated 4175 cells; §p<0.05 compared to vehicle-treated 1833 cells. In B, C, D: **p<0.01, ***p<0.001 compared to the corresponding vehicle-treated cells; $^{#}$p<0.05, $^{##}$p<0.01, $^{###}$p<0.001 compared to the corresponding S1P-stimulated cells.

cells overexpressing $S1P_3$, which overproduce PGE_2 in the presence of S1P and aza-C (Fig. 7E), the migratory response did not markedly increase compared to the untransfected cells. This indicates that in parental MDA-MB-231 cells additional defects are involved which negatively affect migration.

2.3.5. PGE_2 stimulates migration and invasion of metastatic breast cancer cells by EP_2 and EP_4 activation

To investigate the role of the EP subtypes in PGE_2-stimulated cell migration, we measured mRNA expression of the four known receptor EP_{1-4}. EP_2 was highly upregulated in both metastatic sublines with the highest level measured in 1833 cells (Fig. 8A). Expression of the other receptor subtypes was much lower and similar in the three cell lines, except for EP_4, which was slightly higher in 1833 cells (Fig. 8A).

To investigate whether the highly expressed EP_2 is involved in the pro-migratory action of PGE_2 in metastatic 4175 cells, we used antagonists selective for all four receptor subtypes. Fig. 8B shows that antagonism of EP_2 (by PF04418948) and EP_4 (by AH23848) attenuated S1P-induced migration. In contrast, an EP_1 antagonist (SC51322) and an EP_3 antagonist (L-798.106) were inefficient. This indeed suggests a pro-migratory stimulation loop initiated by S1P binding to $S1P_3$, which results in PGE_2 overproduction and secretion

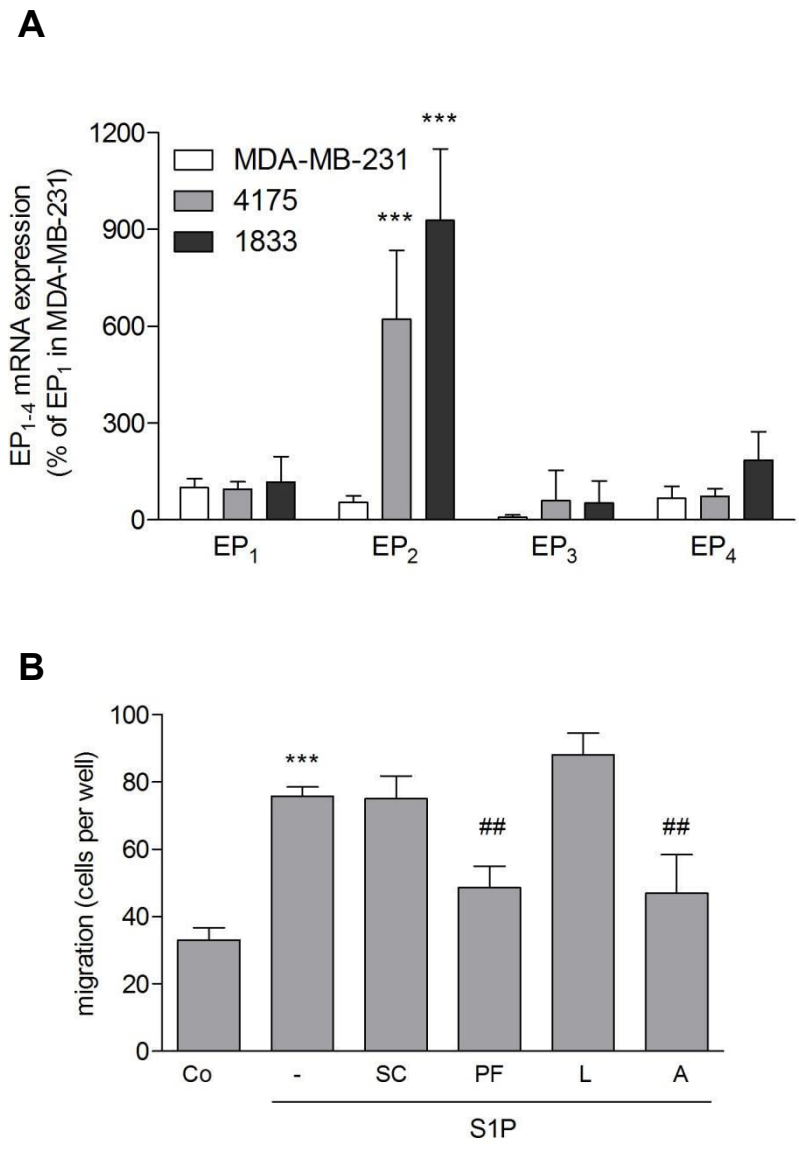


Fig. 8. mRNA expression of EP_{1-4} in parental and metastatic MDA-MB-231 cells and effect of EP antagonists on S1P-induced migration. (A) Cells were cultured for 24 h in serum-free medium before RNA was extracted for PCR analysis of the PGE_2 receptor subtypes EP_{1-4}. 18S RNA was used for normalisation. Data are the mean ± SD (n=6), ***p<0.0001. (B) 2 x 10^5 4175 cells were seeded on transwell filters and treated for 20 h with vehicle control (Co) or S1P (1 µM) in the absence (-) or presence of the EP_1 antagonist SC51322 (SC, 5 µM), the EP_2 antagonist PF04418948 (PF, 5 µM), the EP_3 antagonist L-798.106 (L, 5 µM) or the EP_4 antagonist AH23848 (A, 5 µM). Migrated cells were counted as described in the Materials and Methods section. Data are expressed as number of cells migrated to the lower chamber cells and are the mean ± SD (n=3). **p<0.01 compared to the corresponding vehicle-treated control cells; $^{\#\#}p < 0.01$ compared to the corresponding S1P-stimulated cells.

followed by activation of EP_2 and EP_4. The low level of EP_2 expression on parental MDA-MB-231 cells might explain why $S1P_3$ overexpression and aza-C could only partially increase the migratory potential (Fig. 7D)

Finally, as another hallmark of metastasis we examined the invasive potential of the MDA-MB-231 cell lines resulting from EP_2 and EP_4 signalling. To this end, parental and metastatic cells were allowed to migrate through a membrane covered with Matrigel in a transwell system. Similar to migration, the metastatic cells showed stronger invasion compared to the parental cells and it further increased significantly upon stimulation with S1P or PGE_2 (Fig. 9A). Moreover, as shown in Fig. 9B for the metastatic 4175 subline, the selective $S1P_3$ antagonist TY52156, the COX-2 inhibitor celecoxib and the EP_2 and EP_4 antagonists (PF04418948 and AH23848, respectively) all significantly offset the stimulatory effect of S1P on invasion. Together with the data from the migration assay, this suggests a crucial role of EP_2 and EP_4 in the pro-metastatic activity of PGE_2 in breast cancer.

2.4. Discussion

Inflammatory breast cancer is a metastatic disease characterized by an aggressive and invasive phenotype [5,6]. To investigate the role of S1P and its cognate receptors in inflammation and metastasis, we used a breast cancer metastases model based on the MDA-MB-231 cell line and the two sublines 4175 and 1833 established from single cell-derived progenies with enhanced metastatic ability to lung and bone, respectively, by in vivo selection [34,35]. All three cell lines express a common poor-prognosis gene expression signature, demonstrating that the metastatic clones already pre-exist in the parental cell population, which is derived from a pleural effusion, but in addition show a gene expression profile characteristic for their organ-specific localization

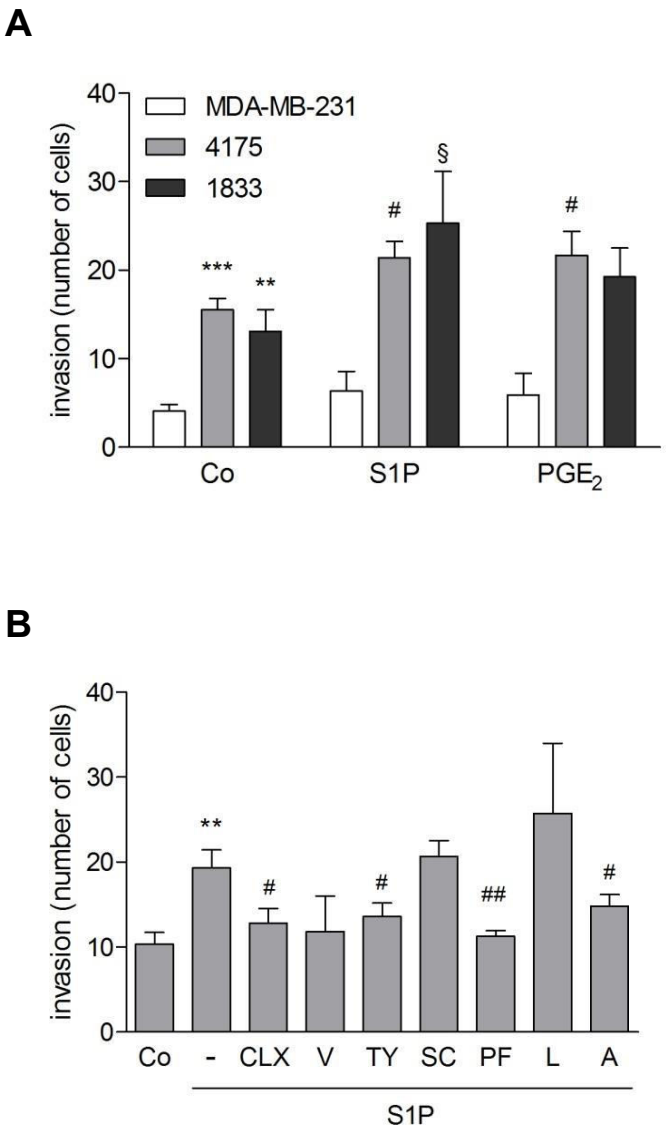


Fig. 9. Effect of S1P and PGE_2 stimulation on invasion of parental and metastatic MDA-MB-231 cells. (A) 2 x 10^5 cells were seeded onto Matrigel-coated transwell filters and treated for 48 h with either vehicle control (Co), S1P (1 µM) or PGE_2 (1 µM). (B) 2 x 10^5 4175 cells were seeded onto Matrigel-coated transwell filters and treated for 48 h with vehicle control (Co) or S1P (1 µM) in the absence (-) or presence of celecoxib (CLX, 5 µM), the $S1P_{1/3}$ antagonists VPC23019 (V, 10 µM) and TY52156 (TY, 1 µM), the EP_1 antagonist SC51322 (SC, 5 µM), the EP_2 antagonist PF04418948 (PF, 5 µM), the EP_3 antagonist L-798.106 (L, 5 µM) or the EP_4 antagonist AH23848 (A, 5 µM). Invaded cells were determined as described in the Materials and Methods section. Data are expressed as number of cells migrated through the Matrigel-coated membrane into the lower chamber and are the mean ± SD (n=3). In A: **p<0.01, ***p<0.001 compared to vehicle-treated parental MDA-MB-231 cells; $^{\#\#\#}$p<0.001 compared to vehicle-treated 4175 cells; §p<0.05 compared to vehicle-treated 1833 cells. In B: **p<0.01 compared to vehicle-treated 4175 cells; $^{\#}$p<0.05, $^{\#\#}$p<0.01, compared to S1P-treated 4175 cells.

[34,35]. The selection of such pre-existing highly metastatic clones from a parental cell population offers the possibility to compare cells with a very similar genetic background and specifically identify differences associated with increased metastasis. Here, we investigated such differences with focus on S1P receptor signaling as this pathway was shown to be implicated in inflammatory processes and to support pro-metastatic features like migration and invasion [20]. We did not further explore differences related to the site of metastasis of the MDA-MB-231 sublines, i.e. lung and bone. Interestingly, we found that the three cell lines revealed distinct profiles of $S1P_3$ receptor expression, which was much higher in both metastatic sublines compared to the parental cell population. We are unable to present data on the protein level as several commercially available $S1P_3$ antibodies revealed irrelevant unspecific bands by Western blotting (data not shown). This has recently been reported as a potential serious drawback if antibodies against GPCR proteins are used and may question published data on these targets [41]**.** $S1P_3$ overexpression has not yet been described by transcriptomic analyses of these cell lines so far, and little is known about its implication in breast cancer development and metastatic progression.

$S1P_3$ couples to G_i, G_q and $G_{12/13}$ [23]. Upon S1P binding, one of the fast intracellular responses mediated by $S1P_3/G_q$ is induction of PLC/IP_3 signaling and Ca^{2+} mobilization [30]. $S1P_3$ overexpressed on the metastatic sublines was fully functional and increased $[Ca^{2+}]_i$ upon stimulation with extracellular S1P. Accordingly, the basal level of $[Ca^{2+}]_i$ in these cells is higher compared to the parental cell line, and stimulation with S1P further induced $[Ca^{2+}]_i$ in the metastatic, but not in the parental cell lines. As expected, this response to S1P was mitigated particularly by the $S1P_{1,3}$ antagonist VPC23019 [42] (Fig. S1), corroborating previous findings that $S1P_3$ and $S1P_2$ can both increase Ca^{2+} mobilization [43]. However, we are aware that the antagonists do not fully discriminate between all receptor subtypes and may even dose-dependently

cross-react as partial agonists [42,44], these data thus only indicate a trend and must be considered in the context of the receptor expression profiles. Similarly, without S1P stimulation we also measured a higher baseline of intracellular Ca^{2+} in the presence of VPC23019 in the metastatic sublines, however, we hypothesize that this was due to suppression of the inhibitory activity of G_i-coupled $S1P_3$.

In the metastatic MDA-MB-231 sublines activation of $S1P_3$ also increased COX-2, but not COX-1 expression, and consequently synthesis of the biologically active prostanoid PGE_2, which could be measured in the culture supernatants. Enhanced COX-2 expression is frequently found in invasive breast cancer [13] and was identified as a hallmark and poor prognosis gene expression signature of metastatic MDA-MB-231 cells [34,35]. In line with this finding we also measured constitutively high levels of COX-2 in the two metastatic sublines compared to the parental cells, and expression could be further increased by treatment with IL-1β/Fk, a potent inducer of the enzyme [45]. In contrast, the parental cells expressed only very low levels of COX-2 and also did not respond to IL-1β/Fk. Interestingly, further investigations using the demethylating agent aza-C [46] unveiled COX-2 promoter silencing in the parental cell population, which in the two sublines was apparently suspended during metastatic selection.

PGE_2 is implicated in various pathological processes such as inflammation and cancer, and its role in carcinogenesis and tumor growth is well established [12]. On the other hand, the PGE_2 analog 16,16-dimethyl-PGE_2 was shown to inhibit intestinal tumor formation and growth [47]. Here, we found that in both metastatic MDA-MB231 sublines the high levels of COX-2 correlated with PGE_2 synthesis, whereas PGE_2 was hardly detectable in the parental cell line (Fig. 3A). mPGES-1 is another inflammation-relevant enzyme which is implicated in the synthesis of inducible PGE_2 and its expression is also associated with tumor growth and metastasis [48]. In fact, compared to the

parental MDA-MB-231 cell line we measured also higher levels of IL-1β/Fk-inducible mPGES-1 in the metastatic sublines, suggesting that it also contributes to the higher basal level of PGE_2 synthesis in these cells.

The pro-inflammatory action of S1P may be due to its ability to stimulate COX-2 expression and subsequent synthesis of PGE_2 [25,26,28]**.** Indeed, we found that S1P induced COX-2 expression and PGE_2 synthesis particularly in the metastatic MDA-MB-231 cells, and the use of antagonists selectively binding to the different S1P receptor subtypes unveiled that this effect was due to activation of the overexpressed $S1P_3$. Further evidence for the role of $S1P_3$ in PGE_2 synthesis was provided by $S1P_3$ downregulation using siRNA and pharmacologically using the $S1P_3$ agonist CYM5541 [49], which showed a similar effect as S1P on the metastatic cells. These findings were corroborated by our data from parental MDA-MB-231 cells overexpressing $S1P_3$ upon transfection. However, as discussed above for COX-2 expression, despite very high $S1P_3$ levels, treatment with S1P alone was ineffective in the parental MDA-MB-231 cells and pretreatment with the DNA demethylating agent aza-C was required to stimulate PGE_2 synthesis. This suggests silencing of the COX-2 promoter as an epigenetic mechanism which was reported also for other genes involved in breast cancer metastasis [39]. These findings demonstrate that S1P by activation of $S1P_3$ has potential to induce the COX-2/PGE_2 pathway, thereby contributing to the pro-inflammatory environment in metastatic breast cancer.

The pro-migratory potential of S1P and PGE_2 has been described in various cell types [18,20,50,51]. In addition, in cancer, PGE_2 also stimulates the angiogenic switch and was shown to promote lymphatic invasion of breast cancer cells [14]. As expected from the several rounds of in vivo selection in nude mice [34], compared to the parental MDA-MB-231 cells the metastatic sublines, particularly 4175, exhibited a higher migratory potential and tissue invasion, which further increased in the presence of exogenous S1P or after the treatment with recombinant PGE_2. Using various S1P receptor subtype-selective

antagonists we could confirm that this effect was mediated by $S1P_3$, which is overexpressed exclusively in the two metastatic MDA-MB-231 sublines. This finding is corroborated by recent data from lung cancer cells where knockdown of $S1P_3$ mitigated S1P-induced migration and invasion [52] and from a melanoma metastasis model where $S1P_3$ stimulated motility and invasiveness triggered by stroma-derived S1P [53]. In breast cancer patients high cytoplasmic $S1P_3$ expression in tumors was shown to correlate with shorter survival and nuclear $S1P_3$ expression to correlate with tumor size [54]. In vitro data from other cancer types also suggest different scenarios for this pro-metastatic $S1P_3$-dependent effect, including G_i-mediated activation of the Rho family GTPase Rac [51] or interaction of G_q with MMP-9 [30]. Using the COX-2 inhibitor celecoxib we clearly demonstrate that the COX-2/PGE_2 pathway is implicated in S1P/$S1P_3$-induced migration and invasion of the here used in vivo selected metastatic breast cancer cells.

Similar to $S1P_3$ we found that $S1P_2$ was also involved in PGE_2 synthesis and its antagonism disrupted S1P-induced PGE_2 synthesis. A role of $S1P_2$ in cyclooxygenase 2 expression was previously also reported by others. Thus, Li et al. [55–57] showed that in a Wilms tumor-derived cell line, S1P-stimulated COX-2 expression occurs in a $S1P_2$-dependent manner and that $S1P_2$ is greatly increased in Wilms tumor tissue. Also, in renal mesangial cells, COX-2 induction by S1P was mediated by $S1P_2$ [25,26], whereas Kim et al. [55–57] reported for amnion-derived WISH cells an exclusive regulation of COX-2 by $S1P_{1\ and\ 3}$, but not by $S1P_2$. Interestingly, although $S1P_2$ stimulation also increases PGE_2 synthesis in metastatic MDA-MB-231 cells, in contrast to $S1P_3$ it does not increase migration. This suggests that $S1P_2$ provides additional cellular signals which counteract the pro-migratory potential of $S1P_3$ and PGE_2. One such mechanism could be activation of the Rho-kinase 1 which was reported to be regulated by $S1P_2$ (reviewed in [55–57]). The outcome of increased migration of the metastatic MDA-MB231 sublines compared to the

parental MDA-MB231 cells may thus be the result of $S1P_3$ overexpression which outweighs the anti-migratory function of $S1P_2$. Moreover, in the lung metastatic cell line 4175 we could not confirm that $S1P_2$ downregulates the breast carcinoma metastasis suppressor 1 (Brms1) (data not shown), a response which in other cancer cell types was reported to promote lung metastasis [58].

PGE_2 binds to four EP subtypes, i.e., EP_{1-4} [15,16]. Particularly, EP_2 and EP_4 are often associated with an aggressive and invasive cancer phenotype [17,18] and EP_2 was shown to mediate the pro-angiogenic effect of PGE_2 in a breast cancer metastases model [59]. Both receptors signal through G_s and increase cAMP, which may explain how stimulation of COX-2/PGE_2 increases expression of the CCR7 chemokine. Furthermore, in breast cancer tissues overexpression of COX-2 correlates with lymph node metastasis [14]. We found that the four receptor subtypes were expressed in the parental and the metastatic MDA-MB-231 cell lines though at different levels. EP_2 showed the highest expression and was upregulated in the two metastatic sublines, whereas EP_3 was hardly detectable (Fig. 9A). Using receptor subtype-selective antagonists we identified EP_2 to be mainly responsible for the stimulatory effect of S1P on migration and invasion of the metastatic cells, whereas EP_4 revealed only a moderate effect.

Altogether, our data demonstrate for the first time that $S1P_3$ overexpression is a potential marker of metastatic breast cancer and that its activation by S1P stimulates the pro-inflammatory COX-2/PGE_2 pathway, which uses the EP_2 and EP_4 receptor to promote cell migration and invasion in an auto- and paracrine manner. The finding that COX-2 expression is low in parental MDA-MB-231 cells line but high and inducible in the metastatic sublines, suggests an epigenetic silencing mechanism which becomes suspended during in vivo metastatic selection. We conclude that targeting $S1P_3$ holds promise for the treatment of inflammation-associated metastatic breast cancer.

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Supplementary Data

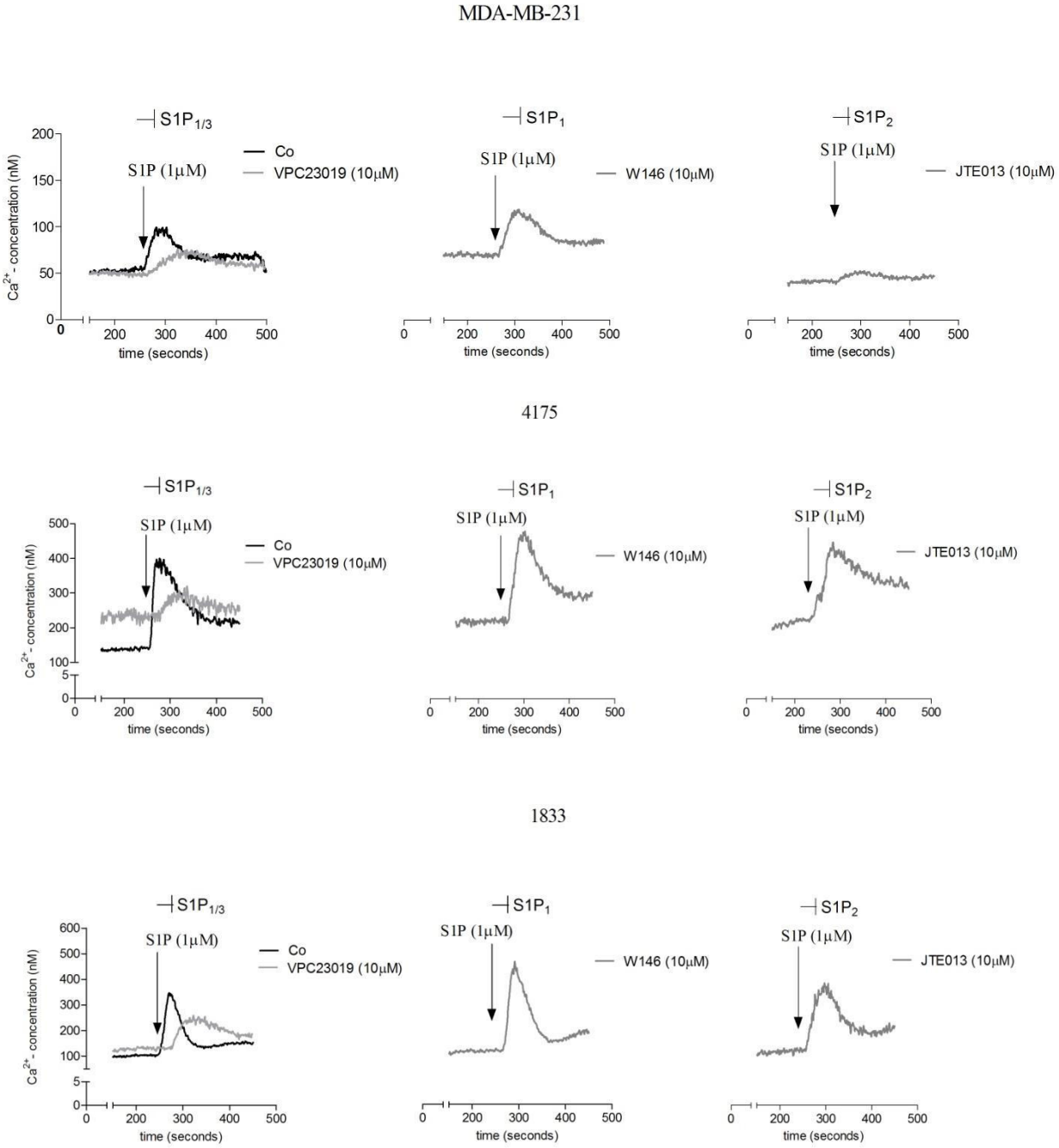

Fig. S1. Effect of different S1PRs antagonists on S1P-induced $[Ca^{2+}]_i$ mobilization in parental and metastatic MDA-MB-231 cells. Cells were loaded with fura-2/AM prior to stimulation with 1 μM S1P in the absence (Co) or presence of the $S1P_{1/3}$ antagonist VPC23019 (V, 10 μM), the $S1P_1$ antagonist W146 (W, 10 μM) or the $S1P_2$ antagonist JTE013 (J, 10 μM). All antagonists were added 10 min prior to S1P stimulation. $[Ca^{2+}]_i$ was measured using a spectrofluorometer as described in the Materials and Methods section. A) Representative curves from parental MDA-MB-231 cells and the metastatic sublines. Results were calculated using the FL WinLab program.

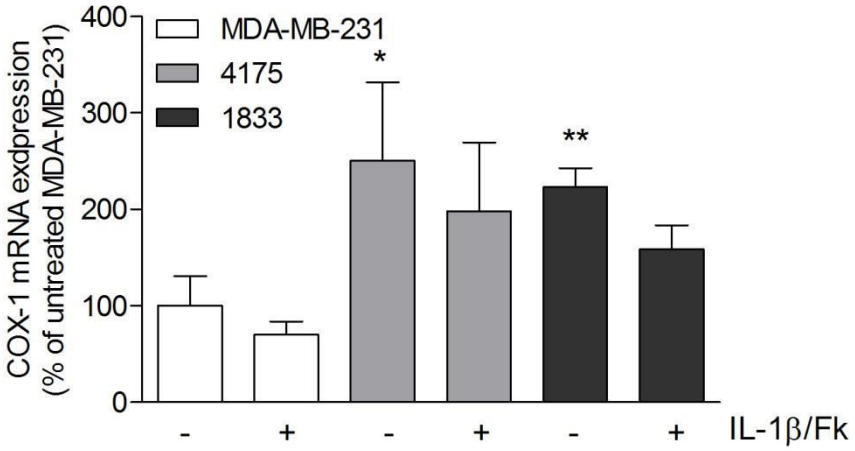

Fig. S2. Expression of COX-1 mRNA in parental and metastatic MDA-MB-231. Cells were incubated for 24 h in serum-free medium prior to stimulation for 24 h with either vehicle control (-) or a combination of IL-1β (1nM) plus forskolin (Fk, 5 μM). Cells were taken for RNA extraction and PCR analysis of COX-1. Data are the mean ± S.D. (n=3). *p<0.05, **p<0.01 compared to unstimulated parental MDA-MB-231 cells

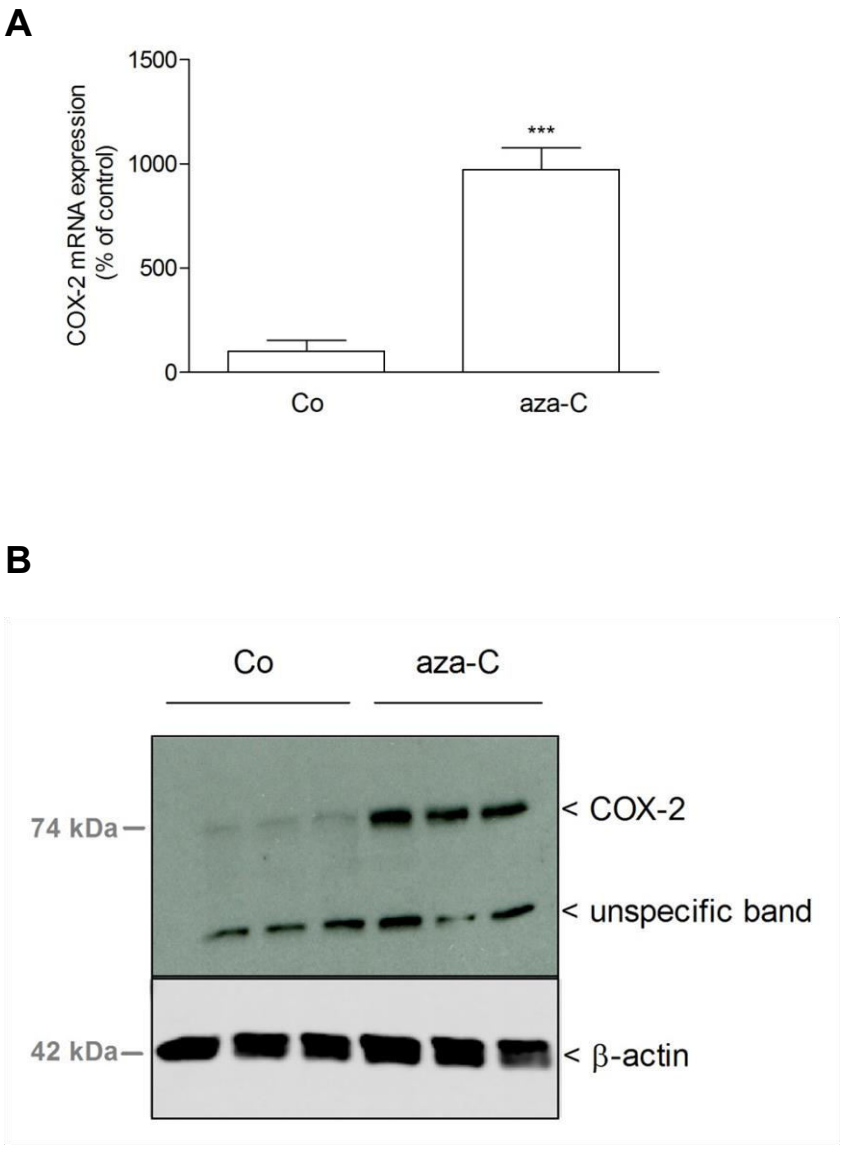

Fig. S3: Effect of the DNA methyltransferase inhibitor aza-C on COX-2 expression in parental MDA-MB-231 cells. Cells were treated for 72 h with either vehicle control (Co) or aza-C (5 µM), which was freshly added every 24 h. Thereafter, cells were collected for RNA extraction and quantitative PCR analysis of COX-2 (A) or were taken for protein extraction, separation by SDS-PAGE (8 %) and Western blotting (B) using antibodies against COX-2 (B, upper panel) and β-actin (B, lower panel), both at dilutions of 1:1000. Data in A are the mean ± S.D. (n=3). $^{***}p<0.001$ compared to vehicle-treated control cells. In B, a representative blot from three independent experiments are shown with samples loaded in triplicates.

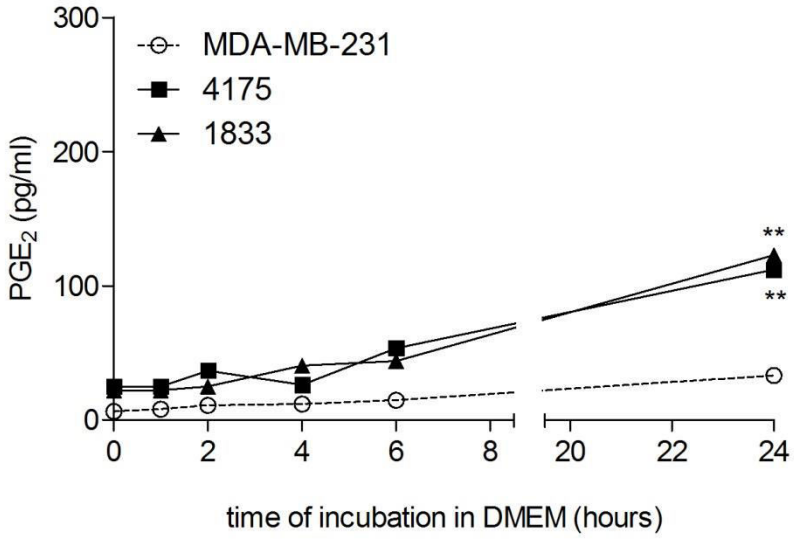

Fig. S4: Basal PGE_2 secretion in the absence of S1P in parental and metastatic MDA-MB-231 cells. Cells were incubated for 1-24 h in DMEM and thereafter, cell supernatants were collected for PGE_2 determination by ELISA. Data are expressed as pg of PGE_2 per ml of supernatant and are the mean ± S.D. (n=3). **p<0.01compared to the parental cell values at 24 h.

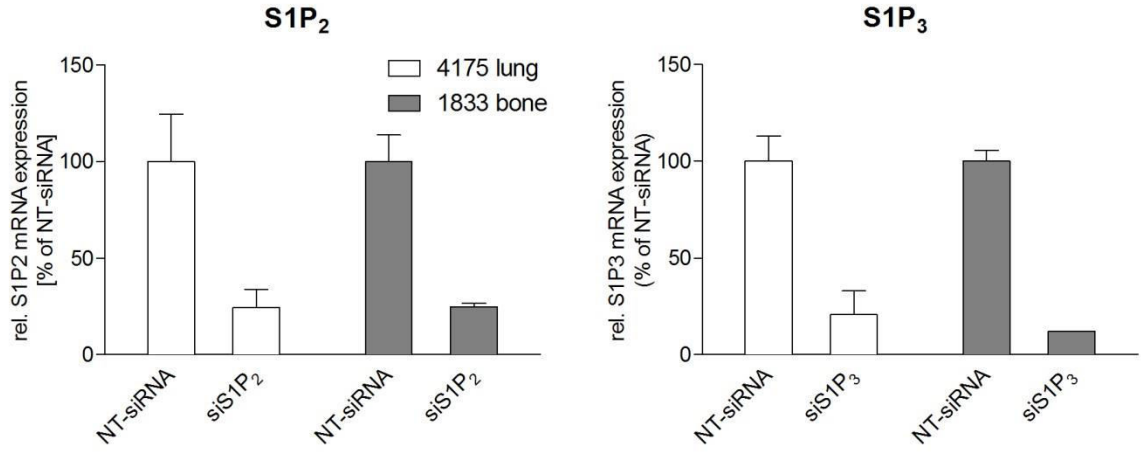

Fig. S5: Downregulation efficiency of siRNA against $S1P_2$ and $S1P_3$ in lung and bone metastatic cell lines. Cells were transfected as described in the Methods Section and thereafter taken for RNA extraction and qPCR analysis of $S1P_2$ (left panel) or $S1P_3$ (right panel). Data are means +/- S.D. (n=3).

Chapter 3

Sphingosine kinase 2 deficiency increasing proliferation and migration of renal mouse mesangial cells and fibroblasts

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Biological Chemistry (2015) 396 (6 – 7): 813 – 825

My particular contribution to this project was in studying the role of $S1P_3$ in proliferation of mMC by using cells isolated from Wt and $S1P_3$ko mice. For this, I performed [^{3}H]Thymidine incorporation assay. Results are presented in Fig. 9A. Another purpose, was to investigate the contribution of $S1P_3$ to migration of mMC by using adapted Boyden chamber assay (data not shown).

Abstract

Both of the sphingosine kinase (SK) subtypes SK-1 and SK-2 catalyse the production of the bioactive lipid molecule sphingosine-1-phosphate (S1P). However, the subtype-specific cellular functions are largely unknown. In this study, we investigated the cellular function of SK-2 in primary mouse renal mesangial cells (mMC) and embryonic fibroblasts (MEF) from wild-type C57BL/6 or SK-2 knockout (SK2ko) mice. We found that SK2ko cells displayed a significantly higher proliferative and migratory phenotype when compared to wild-type cells, with concomitant increased cellular activities of the classical ERK and PI3K/Akt cascades, and of the small G protein RhoA. Furthermore, we detected an upregulation of SK-1 protein and $S1P_3$ receptor mRNA expression in SK-2ko cells. The MEK inhibitor U0126 and the $S1P_{1/3}$ receptor antagonist VPC23019 blocked the increased migration of SK-2ko cells. Additionally, $S1P_3$ko mesangial cells showed a reduced proliferative behaviour and reduced migration rate upon S1P stimulation, suggesting a crucial involvement of the $S1P_3$ receptor. In summary, these data show that SK-2 deficiency in renal mesangial cells and fibroblasts correlates with an enhanced capacity to proliferate and to migrate, indicating that SK-2 exerts suppressive effects on cell growth and migration. Obviously, therapeutic targeting of SKs for treating proliferative diseases essentially requires subtype-selective inhibitors.

3.1. Introduction

One major function of sphingolipids is the structural support of cellular membranes. However, it has become clear that some sphingolipid subspecies also exert important signaling functions and thereby regulate a variety of physiological and pathophysiological processes including cell growth and differentiation, cell survival, migration and inflammation [1–4].

Especially ceramide and sphingosine 1-phosphate (S1P) have attracted a lot of interest, as they build a cellular rheostat that finally determines whether a cell grows or dies [2–4]. These two lipids are rapidly interconverted by the action of two enzyme classes, i.e. the ceramidases that deacylate ceramide to form sphingosine, and sphingosine kinases (SK) that phosphorylate sphingosine to form S1P.

So far, two subtypes of SK, SK-1 and SK-2, have been cloned and characterized [5]. Both subtypes of SK are ubiquitously expressed in most tissues, but show differential subcellular localizations [5]. Although both enzymes catalyse the same reaction and produce S1P, the physiological or pathophysiological functions of the two subtypes and variants are presently not completely clear. Many studies have addressed the functions of SK-1 and there is a broad consent on its key function in cell proliferation and migration [6,7]. This is further supported by studies showing an up-regulation of SK-1 mRNA in several tumors [1,7,8]. Moreover, an increased SK-1 expression correlates with poor survival of breast cancer patients [9].

In contrast, SK-2 has been appointed a rather pro-apoptotic function based on cellular transfection experiments [10]. SK-2 contains a BH3 domain which can interact with Bcl-X_L and thereby inhibit the protective potential of Bcl-X_L consequently resulting in enhanced cell apoptosis. Additionally, the overexpression of SK-2 in various murine and human cell lines resulted in reduced DNA synthesis [11,12]. In line with this, previous data on mouse

mesangial cells isolated from SK-2 knockout mice revealed that lack of SK-2 protected from stress-induced apoptosis [13]. However, recently it was suggested that also SK-2 positively contributes to cancer cell growth, since the putative selective SK-2 inhibitor ABC294640 reduced cancer cell growth in vitro and also in a mouse xenograft model [14].

In the present study, we have used mouse renal mesangial cells (mMC) and mouse embryonic fibroblasts (MEF) isolated from SK-2 deficient mice and investigated changes in cellular responses such as cell migration and proliferation. We show that the loss of SK-2 correlates with a higher proliferative and migratory phenotype of the cells. On the contrary, when using the putative SK-2 inhibitor ABC294640 a reduced proliferation rate of renal mesangial cells was seen which surprisingly, was also detected in SK-2ko cells, suggesting that this compound is non-specific and affects cell proliferation unrelated to SK-2 inhibition. On the molecular level, we found that SK-2 depleted cells have a compensatory upregulated SK-1 protein expression, an increased activity of the small G protein RhoA, increased activities of the classical ERK and PI3K/Akt cascades, and a strongly upregulated $S1P_3$ receptor expression which may all contribute to the increased cell migration and proliferation observed.

3.2. Materials and methods

3.2.1. Chemicals

[6-^{3}H]methyl-thymidine (specific activity: 14.5 Ci/mmol was from American Radiolabeled Chemicals Inc., St. Louis, MO, US; the secondary anti-rabbit and anti-mouse horseradish peroxidase-coupled antibodies, Hyperfilm MP, and the enhanced chemiluminescence (ECL) reagents were from GE Health Care Systems GmbH, Freiburg, Germany; sphingolipid standards for LC/MS, W146, JTE-013 and VPC23019 were from Avanti Polar Lipids Inc.,

Alabaster, AL, US; U0126, and wortmannin were from Merck Biosciences, Schwalbach, Germany; antibodies against phospho-Ser^{473}-PKB/Akt, phospho-ERK1/2, total Akt, and total ERK1/2 were from Cell Signaling, Frankfurt, Germany; the β-actin (clone AC-15) antibody, DAPI, were from Sigma Aldrich Fine Chemicals, St. Louis, U.S.; RhoA G-lisa was from Cytoskeleton Inc., CO, US; all cell culture media and supplements were from Invitrogen, Allschwil, Switzerland.

3.2.2. Peptide synthesis and antibody generation for SK-1 and SK-2 antibodies

Peptide synthesis and antibody generation was performed by Eurogentec s.a. (Seraing, Belgium). Two synthetic peptides based on the sequence of the mouse SK-1 (accession number: NM_011451) (-CPS GRD SRR GPP PEE P-COOH and –LEP RSQ-RGV FSV DGE C-$CONH_2$) or on the sequence of the mouse SK-2 (accession number: NM_020011) (-CTL LTG PAG QKP QA-COOH and -CPI AEG PPE MPA SSG F-$CONH_2$) were synthesized and coupled to keyhole-limpet hemocyamin, and used to immunize two rabbits. The antibodies from terminal bleeds were purified by affinity chromatography by using an anti-peptide-coupled sepharose column.

3.2.3. Cell culturing

Primary cultures of mouse renal mesangial cells were isolated from C57BL/6 mice (Wt) or SK-2 knockout (ko) mice exactly as previously described [13,15]. Outgrown mesangial cells were subcultured and further used up to passage 20. Cells were cultured in RPMI medium containing 15% fetal bovine serum (FBS), 10 mM HEPES, pH 7.4, 100 units/ml penicilline, 100 μg/ml streptomycin, 6 μg/ml bovine insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite, 4.5 μg/ml β-mercaptoethanol. Mouse embryonic fibroblasts (MEF) were isolated from C57BL6 mice or SK-2 deficient mice as previously

described (Conner, 2001). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 10 mM HEPES, pH 7.4, 100 units/ml penicilline, 100 µg/ml streptomycin.

3.2.4. siRNA transfection

siRNA transfection was conducted by using the Amaxa™ Mouse/Rat Hepatocyte Nucleofector™ Kit (Lonza, Cologne, Germany) according to the Amaxa™ protocol for mouse embryonic fibroblasts. Briefly, mouse mesangial cells were harvested by trypsinization, centrifuged and resuspended in 100 µl Nucleofector™ solution. After addition of either 0.5 µM non-target siRNA (Thermo Fisher Scientic, Waltham, MA, USA) or 0.5 µM SK-2 siRNA (antisense sequence: GCC CUA CAC AUA CAG CGA C) transfection was perfomed in a cuvette using protocol N-024. The cells were immediately transferred to the culture plate and cultured at 37°C for 1-2 days prior to further experiments.

3.2.5. Cell stimulation and Western blot analysis

Confluent cells in 100 mm-diameter dishes were rendered quiescent by incubating for 24 h in DMEM containing 0.1 mg/ml of fatty acid-free bovine serum albumin (BSA). Thereafter, cells were treated as indicated. To stop the stimulation, the medium was withdrawn and the cells were homogenized as previously described [16]. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes and subjected to Western blot analysis as previously described [16].

3.2.6. Transwell migration assay

Cell migration was measured by an adapted Boyden chamber assay. In brief, the ability of cells to migrate through a Transwell filter (6.5 mm diameter, 8µM pore size) was analysed. After serum starvation, cells were detached by trypsinization and seeded into transwell filters at 1 x 10^5 cells in 100 µl

starvation medium. 500 µl of starvation medium was placed in the lower compartment, and the cells were left to migrate for the indicated time periods. Thereafter, the Transwell filters were removed and processed exactly as previously described [17]. Migrated cells were determined by counting the DAPI-stained cells on the filters in five random areas per sample using a fluorescence microscope.

3.2.7. Scratch assay for cell migration

Equal number of cells were seeded in growth medium in PS35 and cultured for 6 h to allow adherence followed by a starvation period in DMEM containing 1% of FBS for 18 h. Thereafter, a "scratch" was created on the confluent cell monolayer using a p200 pipet tip and the dish was immediately photographed under a phase-contrast microscope at specific reference points. A second image was taken 24 h after previous alignment along the reference points to ensure assessment of the same scratch area. The images were analysed using the Image J software (Wayne Rasband, NIH, USA) by measuring the scratch area at 0 h and 24 h after incubation. Data are expressed as area reduction from time points 0 to 24 h.

3.2.8. [^{3}H]Thymidine incorporation

5000 cells were plated per well of a 24-well plate and incubated for 20 h. Thereafter, cells were incubated in growth medium containing 0.2 µCi/ml of [^{3}H] methyl-thymidine. After different time periods, the medium was removed and cells were washed twice with PBS, and incubated with ice-cold 5% trichloroacetic acid (TCA) for 30 min. Cells were washed twice with 5% TCA and the DNA was solubilized in 1 M NaOH for 30 min at 37°C, and the radioactivity was counted in a β-counter.

3.2.9. Quantitative PCR analysis

Real-time PCR was performed using SYBRgreen® and a BioRad iQ iCycler Detection System. Primer sequences were as follows in table 3.1.

Table 3.1. Characteristic of primers used for qPCR analysis

Gene	Forward primer (from 5' to 3') Reverse primer (from 5' to 3')
mouse $S1P_1$	CTGACCTTCCGCAAGAACATCT CTTCAGCAAGGCCAGAGACTTC
mouse $S1P_2$	GAGCTCATCACCTCTTCATCCTATC GAAGATGCAGTAAGAGTACCCAGGA
mouse $S1P_3$	TGTAGCTTCATCGTCGTCTTGGAG GCCGATGAAAAAGTACATGCGG
mouse $S1P_4$	GGCTACTGGCAGCTATCCTG AAGGCCACCAAGATCATCAG
mouse $S1P_5$	GGAGGGACTCTCCTGGATTC TTCCTCTGTAGCCAGCCACT

IQ™5 Optical System Software (Version 2.0) was used to analyze real time and endpoint fluorescence. One microgram of total RNA isolated with TRIZOL® reagent was used for reverse transcriptase–PCR (First Strand Synthesis Kit, MBI Fermentas, St-Leon-Roth, Germany); a random hexamer primer was utilized for amplification. The fold induction values were obtained according to the $\Delta\Delta C_T$ method, after normalization to the housekeeping gene 18S RNA.

3.2.10. RhoA activity assay

The activity of the small G protein RhoA was determined by using a specific G-LISA™ activity assay kit (Cytoskeleton Inc., Denver, CO, US) exactly as described in the manufacturer's manual.

3.2.11. Lipid quantification by mass spectrometry

Confluent cells in PS35 wells were either taken for lipid extraction and LC-MS/MS analysis exactly as previously described [13] or taken for cell counting for lipid equalization.

3.2.12. Statistical analysis

Statistical analysis was performed by unpaired t-test and two-tailed p values for the comparison of two groups, and by one-way analysis of variance (ANOVA) with Bonferroni post test for the comparison of three and more groups.

3.3. Results

3.3.1. Loss of SK-2 leads to increased proliferation and migration of mouse mesangial cells and fibroblasts.

Since the cellular function of SK-2 is still poorly understood, we here used renal mesangial cells and mouse embryonic fibroblasts isolated from either wild-type mice or SK-2 gene deficient mice to unravel a possible contribution of SK-2 to physiological or pathophysiological cell responses. First, renal mesangial cells (Fig. 1A) and mouse embryonic fibroblasts (Fig. 1B) were characterized for SK-2 protein expression by Western blot analysis. SK-2 runs as an approx. 68 kDa band in SDS-PAGE. In primary cultures of mMCs or MEFs isolated from SK-2 knockout (ko) mice, this band was abolished confirming the complete knockout of the gene. The antibody additionally recognized a band at 43 kDa, which was not reduced in the SK-2 depleted cells, thus suggesting a likely unspecific staining.

Under normal cell culturing conditions, it became very obvious that SK-2ko cells proliferated much faster than the corresponding wild-type cells. To

confirm that DNA synthesis was enhanced, [^{3}H] thymidine incorporation was measured in cells plated in equal numbers. As seen in Fig. 2A, over the time period of 2 days, SK-2ko mMC showed a several fold increase of DNA synthesis. A similar increase was also seen in SK-2ko MEFs when compared to the wild-type cells (Fig. 2B) and in mesangial cells that were transiently transfected with siRNA against SK-2 (Fig. 2C). Additionally, we tested the putative SK-2 inhibitor ABC294640 on renal mesangial cell proliferation and detected a reduction of cell proliferation at a concentration of 30 µM and even more pronounced at 100 µM. Strikingly, the same effect was obtained when using SK-2 knockout mesangial cells (Fig. 2D, grey bars) suggesting an unspecific effect of ABC294640 not related to SK-2 inhibition.

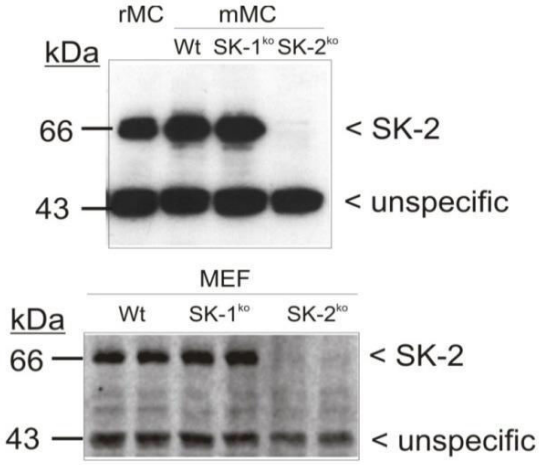

Figure 1. Sphingosine kinase 2 protein expression in mouse renal mesangial cells and embryonic fibroblasts. Protein lysates of quiescent mouse mesangial cells (mMC) and mouse embryonic fibroblasts (MEF) isolated from either C57BL/6 mice (Wt), SK-1 deficient mice (SK-1ko), or SK-2 deficient mice (SK-2ko), or rat mesangial cells (rMC) were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis using a specific antibody against mouse SK-2 at a dilution of 1:1000. Bands were visualised by the ECL method according to the manufacturer's instructions.

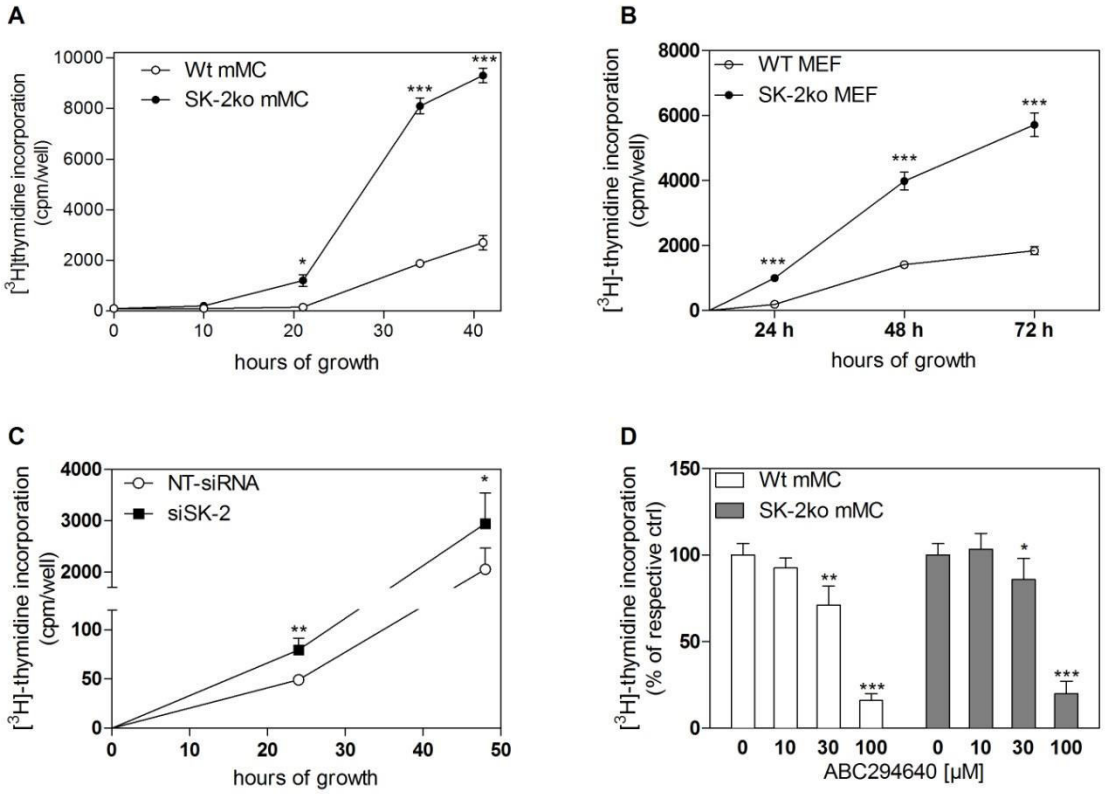

Fig.2. Effect of SK-2 deficiency on proliferation of mouse mesangial cells and embryonic fibroblasts. 10^4 mouse mesangial cells (A, C, D, mMC) or mouse embryonic fibroblasts (B, MEF) isolated from either wild-type C57BL/6 mice (Wt, open circles) or SK-2 deficient mice (SK-2ko, closed circles) or mesangial cells transiently transfected with a non-target siRNA (C, NT-siRNA, open circles) or siRNA against SK-2 (C, siSK-2, closed circles) were plated and grown for the indicated time periods in growth medium supplemented with [^{3}H]thymidine alone (A, B, C) or with the indicated concentrations of ABC294640 for 24 h (D) and processed as described in the Methods Section. [^{3}H]thymidine incorporated into DNA was measured in a β-counter. Data are expressed as cpm/well (A, B) or % of control (C, D) and are means ± S.D. (n=3-5). *p<0.05, **p<0.01, ***p<0.001 considered statistically significant compared to the corresponding Wt values (A, B) or to the respective control values (C, D).

In a next step, the migratory capacity of cells was determined. For this, either an adapted Boyden chamber assay (Fig. 3A), or a scratch assay (Figs. 3B-G) was performed. In the adapted Boyden chamber assay, equal numbers of cells were plated onto a transwell filter with 8µm pores and were allowed to migrate through the filter for 7h. Thereafter, non-migrated cells were scraped

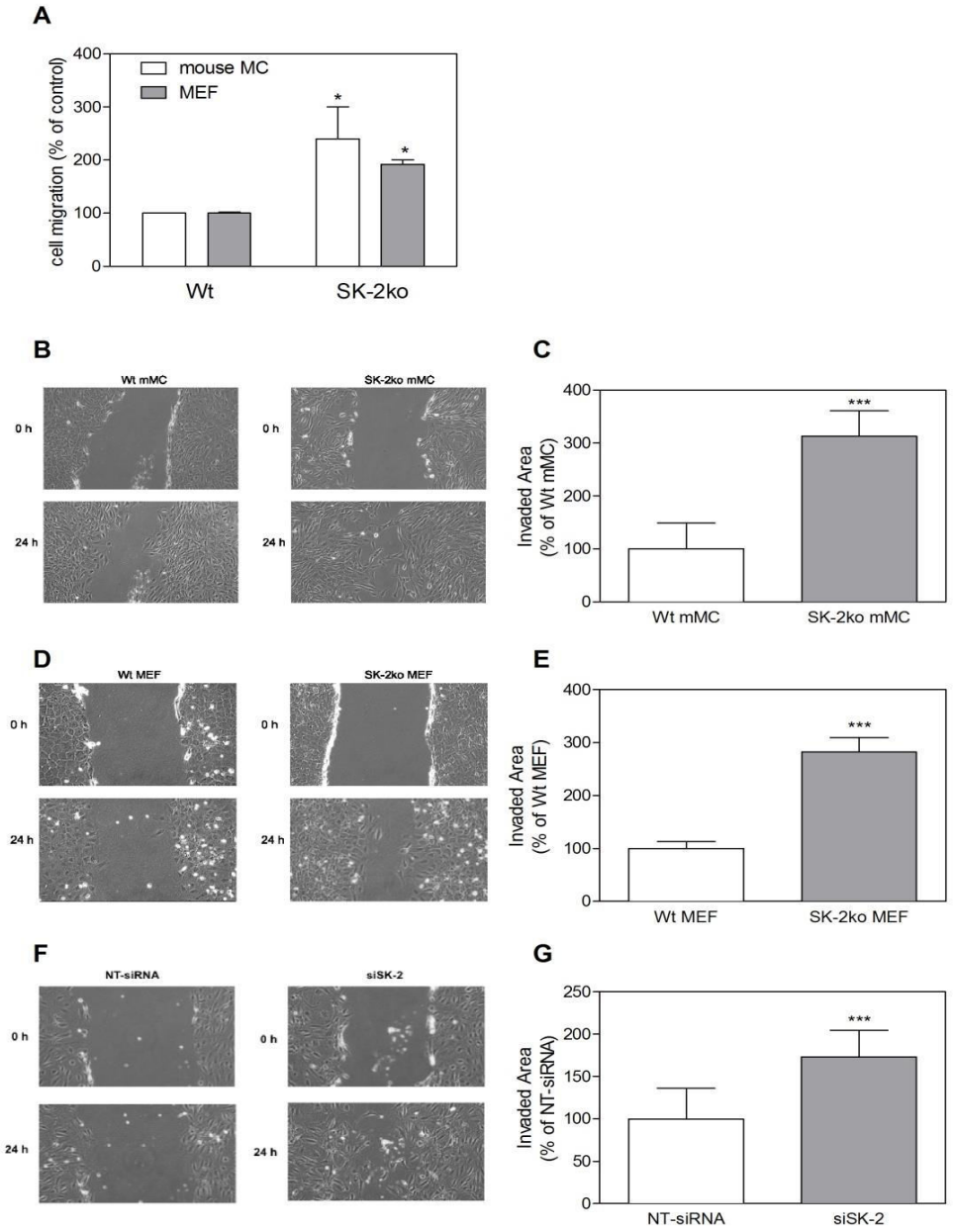

Fig. 3: Effect of SK-2 deficiency on migration of mouse mesangial cells and embryonic fibroblasts. (A) 10^5 serum-starved mouse mesangial cells or mouse embryonic fibroblasts isolated from either wild-type (Wt) or SK-2 deficient mice (SK-2ko) were subcultured onto transwell filters and allowed to migrate for 7h. Migrated cells were determined as described in the Methods Section. (B - G) Quiescent cells were subjected to a scratch and then allowed to recover for 24 h in DMEM containing 1% FBS. Light microscopy pictures were taken at time point 0 h and 24 h after the scratch. Data in C, E, G show the quantification of the scratch and are expressed as invaded area from time point 0 h to 24 h as % of the Wt or NT-siRNA control and are means ± S.D. (n=6-9). ***$p<0.001$ considered statistically significant compared to the Wt or the NT-siRNA control.

away from the surface of the filter and cells that had migrated into the filter were visualised by DAPI staining. Fig. 3A shows that again, SK-2ko cells migrated faster than wild-type cells. Another method to measure cell migration is the so-called scratch assay. However, this assay can not discriminate between migrating cells and proliferating cells and results reflect more a mixture of migration and proliferation. Therefore, to minimize the amount of cell proliferation, we performed this assay under strongly reduced serum concentrations (1% FBS). Again, our data revealed that SK-2ko mesangial cells (Fig. 3B, right panels) and SK-2ko MEFs (Fig. 3D, right panels) or cells transfected with siRNA against SK-2 (Fig. 3F, right panels) migrated faster than the corresponding wild-type (Figs. 3B and 3D, left panels) or non-target siRNA transfected cells (Fig. 3F, left panels).

3.3.2. Loss of SK-2 leads to activation of ERK and Akt signalling in mesangial cells and fibroblasts

We further investigated whether the increased proliferation and migration of SK-2ko cells was due to specifically activated signaling cascades such as the well known proliferation and migration regulating classical mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) cascade. To this end, Western blot analyses of mesangial cell lysates were performed by staining for phosphorylated and thus activated p44-ERK1 and p42-ERK2. Quiescent wild-type cells which were incubated for 6 h in a very low serum concentration (1% FBS) expressed low levels of phospho-ERK1/2, whereas the SK-2ko cells showed a significantly increased ERK1/2 phosphorylation (Fig. 4A). Another signaling cascade that has been linked to cell growth and survival, but also to cell migration, is the PI3K/Akt cascade [18]. We found that also phospho-Akt was enhanced in SK-2ko mesangial cells (Fig. 4B). Similar data were obtained in MEFs (Fig. 4C and 4D). All these data suggest that in SK-2ko cells, the classical MAPK/ERK and the PI3K/Akt cascades are hyperactivated.

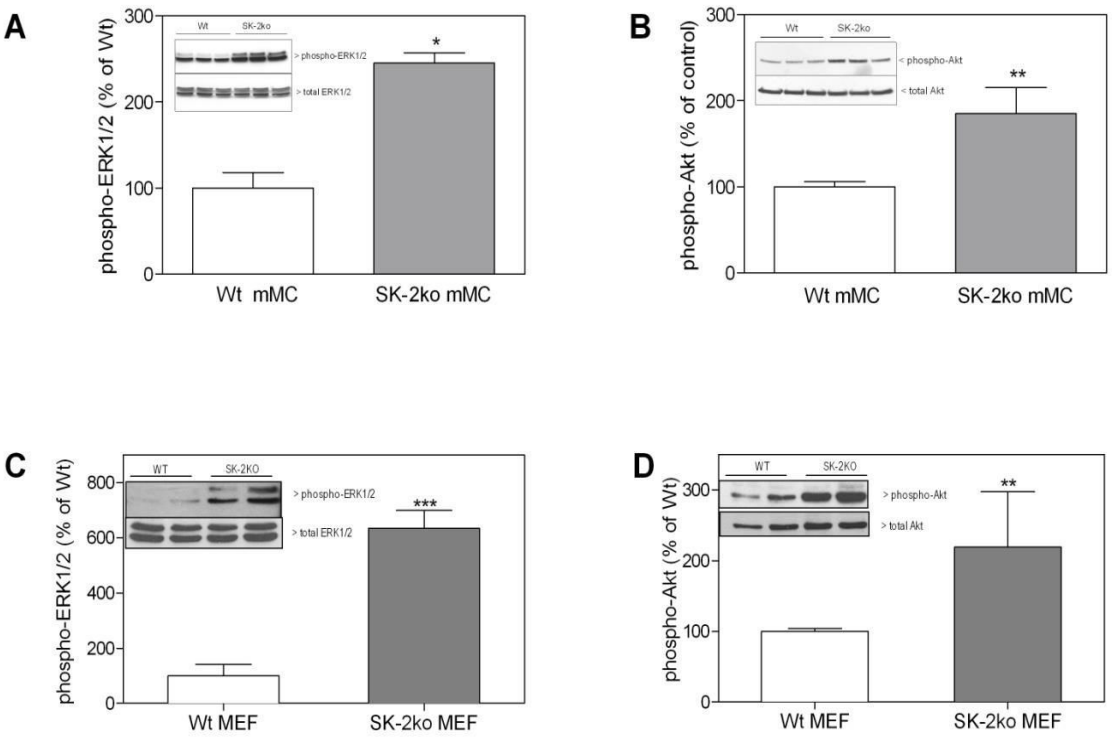

Fig. 4: Effect of SK-2 deficiency on the activation state of ERK1/2 and PKB/Akt in mouse mesangial cells and fibroblasts. Quiescent mouse mesangial cells (A and B) and mouse embryonic fibroblasts (C and D) isolated from either wild-type (Wt, white bars) or SK-2 deficient mice (SK-2ko, grey bars) were incubated for 6 h in DMEM containing 1% FBS. Thereafter, cell lysates containing 50 µg of protein were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis using antibodies against phospho-ERK1/2 and total ERK1/2 (A and C), and phospho-Ser^{473}-PKB/Akt and total PKB/Akt1 (B and D). Bands were visualized by the ECL method according to the manufacturer's instructions and bands were densitometrically evaluated. Data are expressed as % of controls and are means ± S.D. (n=3). *p<0.05, **p<0.01, ***p<0.001 considered statistically significant compared to the corresponding Wt values. The insets show the corresponding immunoblots.

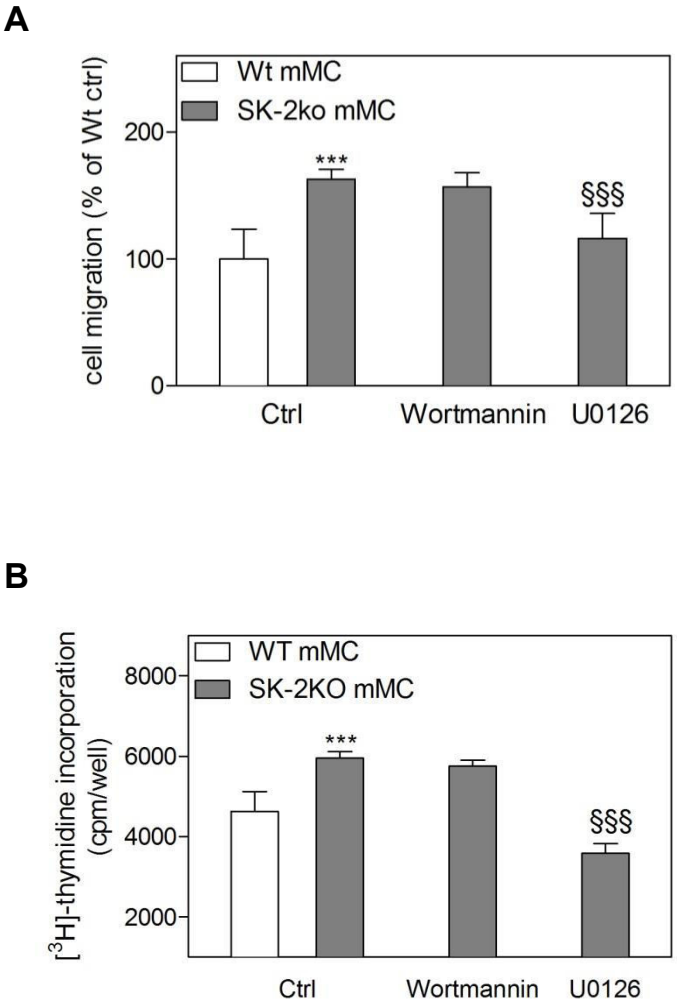

Fig. 5: Involvement of the PI3K and ERK pathway in the SK2ko-mediated increased migration and proliferation in mesangial cells. (A) Quiescent wild-type (Wt, white bar)) or SK-2 deficient (SK-2ko, grey bars) mesangial cells (mMC) were subjected to a scratch and then allowed to recover for 24 h in DMEM containing 1% FBS in the absence (Ctrl) or presence of wortmannin (200 nM) or U0126 (10 µM). Light microscopy pictures were taken at time point 0 h and 24 h after the scratch and analysed using the Image J software (Wayne Rasband, NIH, USA) by measuring the scratch area. Data are expressed as area reduction from time points 0 h to 24 h as % of the Wt control and are means ± S.D. (n=4). (B) Wild-type (Wt, white bars) or SK-2 deficient (SK-2ko, grey bars) mesangial cells (mMC) were plated and grown for the indicated time periods in growth medium supplemented with [^{3}H]thymidine alone (Ctrl) or in the presence of wortmannin (200 nM) or U0126 (10 µM) and processed as described in the Methods Section. [^{3}H]thymidine incorporated into DNA was measured in a β-counter. Data are expressed as cpm/well and are means ± S.D. (n=4-6). ***$p<0.001$ considered statistically significant compared the Wt ctrl, $^{§§§}p<0.001$ considered statistically significant compared to the SK-2ko ctrl value.

To see whether the ERK and PI3K/Akt pathways, are involved in the increased migration and proliferation seen in Sk-2ko cells, we performed scratch assays and measured DNA synthesis of mouse mesangial cells in the absence or presence of inhibitors of these signalling pathways. Fig. 5A and 5B show that the specific MEK inhibitor U0126 [19,20], but not the PI3K inhibitor wortmannin [20] inhibited migration and proliferation of SK-2ko mMC.

Furthermore, it is well known that small G proteins are important molecular regulators of cell migration and also proliferation [21]. We therefore investigated whether small G proteins are altered in SK-2ko cells. Small G protein activity was measured by G-LISA®, which specifically detect the activated forms of the indicated small G proteins [22]. As seen in Fig. 6, basal RhoA activity was significantly increased in SK-2ko mesangial cells and this was further enhanced after S1P treatment. By contrast Ras, Rac and cdc42 were not altered in SK-2ko mesangial cells (data not shown).

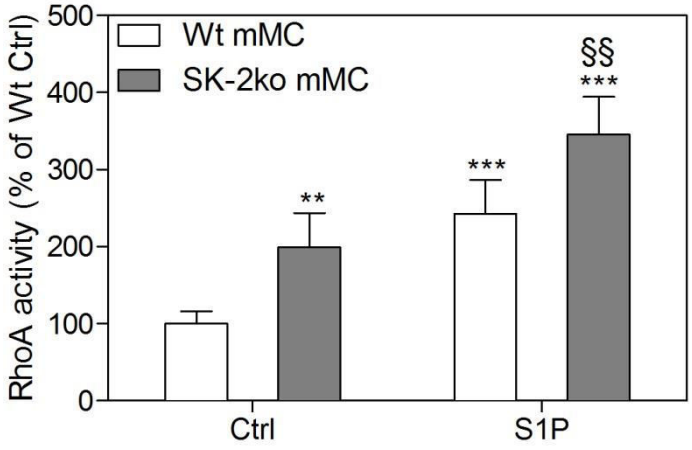

Fig. 6: Effect of SK-2 deficiency on RhoA activity in mouse mesangial cells. Quiescent mouse mesangial cells isolated from either wild-type (Wt, white bars) or SK-2 deficient mice (SK-2ko, grey bars) were stimulated for 10 min with either vehicle (Ctrl) or sphingosine 1-phosphate (S1P, 1 µM) in the presence of 1% FBS. Thereafter, cell lysates were subjected to G-LISA® RhoA activation assay according to the manufacturer´s instructions. Data are expressed % of Wt ctrl and are means ± S.D. (n=3). **p<0.01, ***p<0.001 considered statistically significant compared to the vehicle treated Wt value, §§p<0.01 considered statistically significant compared to the S1P-stimulated Wt value.

3.3.3. Loss of SK-2 is associated with enhanced expression of SK-1 and the $S1P_3$ receptor.

In a next step, we tested both cell types for a possible compensatory upregulation of SK-1 expression. By using Western blot analysis, we indeed found an enhanced expression of SK-1 protein in SK-2ko mesangial cells and MEF. Even a partial and transient reduction of SK-2 with SK-2-siRNA resulted in a higher SK-1 protein expression compared to non-target siRNA transfected cells (Fig. 7A). Furthermore, we screened the cells for S1P receptor subtype expression and interestingly found that in SK-2ko mMCs (Fig. 7B) and MEFs (Fig. 7C), specifically the $S1P_3$ was highly up-regulated on mRNA level. In addition, we quantified cellular S1P in the cells and found that SK-2ko MEF contain significantly more cellular S1P than the wild-type cells (Fig. 7E). Despite a similar upregulation of SK-1 protein in SK-2ko mesangial cells, the S1P concentration in mMCs did not vary between wild-type and SK-2ko cells (Fig. 7D). Sphingosine and C16-ceramide accumulated in SK-2ko cells in both cell types (Fig. 7D, E). To address if the altered S1P receptor expression profile had an impact on cell motility, we performed a scratch migration assay in the presence of the S1P receptor antagonists W146 ($S1P_1$ antagonist), JTE-013 ($S1P_2$ antagonist) and VPC23019 ($S1P_{1/3}$ antagonist). Our data revealed that a significant reduction of cell migration was only detected in SK-2ko cells when using the $S1P_{1/3}$ inhibitor VPC23019, but not in the presence of W146, thereby excluding a role of $S1P_1$ in the increased migration capacity of SK-2ko cells (Fig. 8). The $S1P_2$ inhibitor JTE-013 enhanced the migratory response of wild-type and SK-2ko cells, thereby demonstrating a potentially inhibitory role of $S1P_2$ in mesangial cell migration (Fig. 8). These data make it tempting to speculate that the observed enhanced migration and proliferation of SK-2ko cells may be due to an autocrine S1P action through the $S1P_3$ receptor, although a further increase of S1P levels in renal mesangial cells seems dispensable.

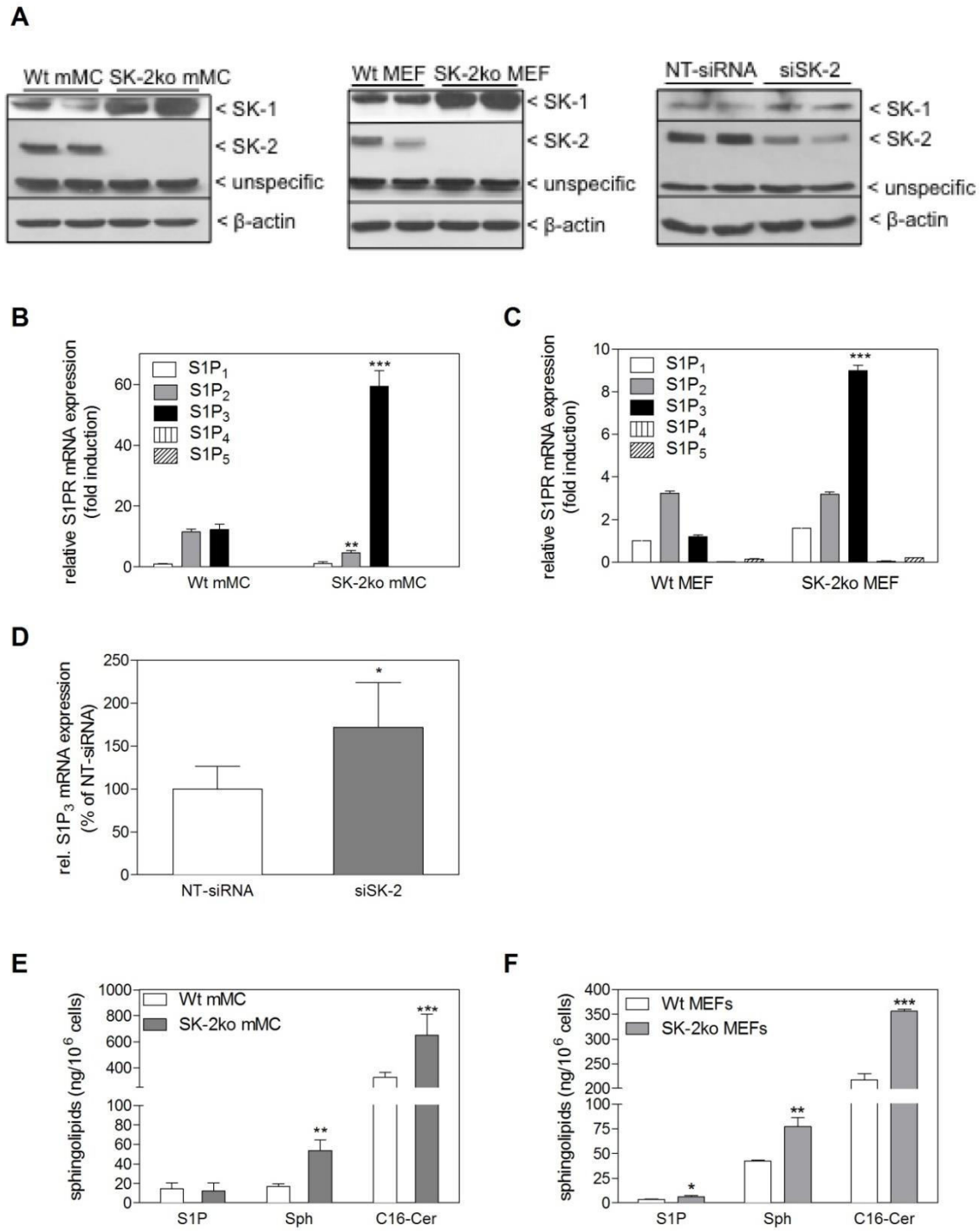

Fig. 7: Effect of SK-2 deficiency on SK-1 protein expression, S1P receptor subtype mRNA expression and on cellular sphingolipid levels in mouse mesangial cells and embryonic fibroblasts. ((A) Quiescent mouse mesangial cells and mouse embryonic fibroblasts isolated from either C57BL/6 mice (Wt) or SK-2 deficient mice (SK-2ko) or mouse mesangial cells transiently transfected with either non-target siRNA (NT-siRNA) or siRNA against SK-2 (siSK-2) were subjected to WB analysis using specific antibodies against SK-1 (upper panels), SK-2 (middle panels) or β-actin (lower panels). (B–D) Quiescent mouse mesangial cells (B) and mouse embryonic fibroblasts (C) isolated from either C57BL/6 mice (Wt) or SK-2 deficient mice (SK-2ko) or

transiently transfected mouse mesangial cells with either non-target siRNA (D, NT-siRNA) or siRNA against SK-2 (D, siSK-2) were taken for RNA extraction and subjected to quantitative PCR analysis using mouse primers of S1P1-5 (B, C) or S1P3 (D) as described in the Methods section. Data were obtained according to the ΔΔ*C*T method and are expressed as fold induction of S1P1 expression level (B, C) or as% of NT-siRNA (D) and are mean• }SD (n = 3–4). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 considered statistically significant compared to the corresponding Wt values. (E, F) Lipids were extracted from quiescent mouse mesangial cells (E) and mouse embryonic fibroblasts (F) from wild-type (Wt, white bars) and SK-2 deficient mice (SK-2ko, grey bars) and taken for LC-MS/MS to quantify sphingosine 1-phosphate (S1P), sphingosine (Sph) and C16-ceramide (C16-Cer). Results are expressed as ng lipid per 106 cells and are mean• }SD (n = 3). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 considered statistically significant compared to the corresponding Wt values.

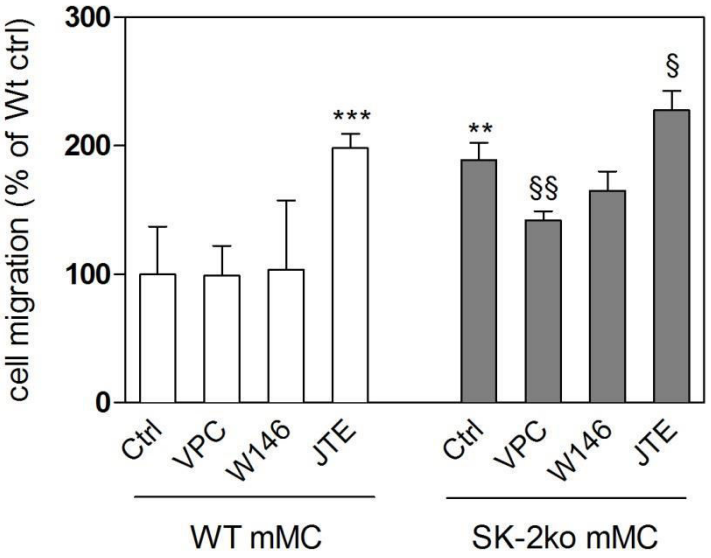

Fig. 8: Effect of S1P receptor antagonists on the increased migration of mouse mesangial cells. Quiescent wild-type (Wt, white bars) or SK-2 deficient (SK-2ko, grey bars) mesangial cells (mMC) were subjected to a scratch and then allowed to recover for 24 h in DMEM containing 1% FBS in the presence of either vehicle (Ctrl) or VPC23019 (VPC, 10 µM), W146 (10 µM) or JTE-013 (JTE, 10 µM). Light microscopy pictures were taken at time point 0 h and 24 h after the scratch and analysed using the Image J software (Wayne Rasband, NIH, USA) by measuring the scratch area. Data are expressed as area reduction from time points 0 h to 24 h as % of the Wt ctrl and are means ± S.D. (n=4). **p<0.01, ***p<0.001 considered statistically significant compared to the Wt ctrl, §§p<0.01 considered statistically significant compared to the SK-2ko ctrl value.

Finally, we investigated the contribution of $S1P_3$ to mesangial cell proliferation and migration by using cells isolated from $S1P_3$ knockout mice. Data in Fig. 9A show that the growth of $S1P_3$ko mMCs in normal growth medium containing 10% FBS was significantly but only partially reduced when compared to wild-type cells. When migration of these cells was analysed in a scratch assay, we found that stimulation of cells with S1P triggered increased migration in wild-type cells (Fig 9B), which was abolished in $S1P_3$ko cells (Fig 9B). Also the stimulation of RhoA activity by S1P detected in wild-type cells was clearly reduced in $S1P_3$ko cells (Fig. 9C). Together these data suggest that indeed $S1P_3$ is contributing to mesangial cell proliferation and migration.

3.4. Discussion

In this study we show for the first time that renal mesangial cells and embryonic fibroblasts isolated from SK-2 deficient mice have a higher capacity to proliferate and migrate when compared to wild-type cells. Since the successful cloning of SK-2 in 2000 [23], the few published studies have reported rather controversial data on possible cellular functions of SK-2. On the one hand, overexpression studies in various cell types revealed that SK-2 is localised in the nucleus and can inhibit DNA synthesis [11]. Moreover, Liu et al. reported that overexpression of SK-2 had a pro-apoptotic effect on cells due to the existence of a BH3 domain which interacts with the anti-apoptotic factor Bcl-XL and thereby blocks Bcl-XL's anti-apoptotic function [10]. In line with this, our own previous studies revealed that a loss of SK-2 rendered mesangial cells less sensitive to stress-induced apoptosis [13].

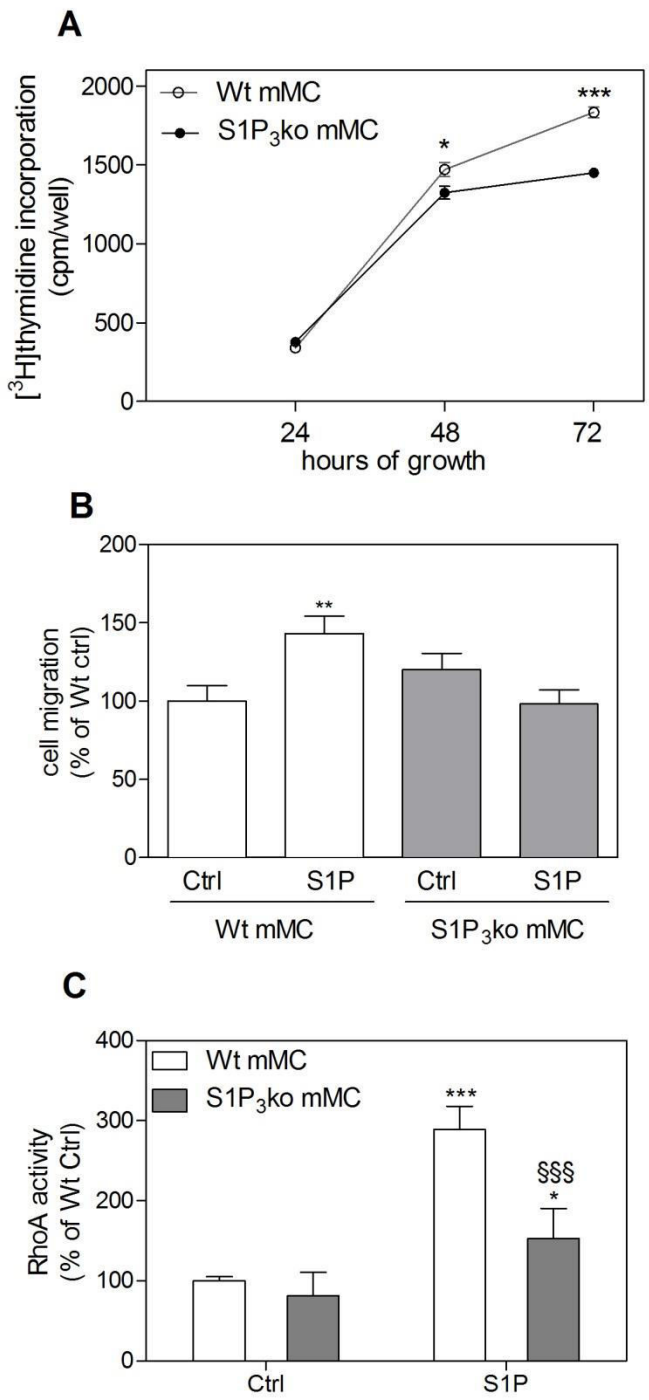

Fig. 9: Effect of $S1P_3$ knockout on proliferation and migration of mouse mesangial cells. (A) 10^5 mesangial cells isolated from either BalbC mice (Wt) or $S1P_3$-deficient mice ($S1P_3$ko) were plated and proliferation assay was processed as described in the Methods Section. [^{3}H]thymidine incorporated into DNA was measured in a β-counter. Data are expressed as cpm/well and are means ± S.D. (n=3). *p<0.05, ***p<0.001 statistically significant compared to the corresponding control values. (B) Quiescent control mouse mesangial cells or $S1P_3$-deficient mesangial cells were subjected to a scratch and allowed to recover for 24 h in DMEM containing 1% FBS the presence of either vehicle (Ctrl) or S1P (1 µM). Light microscopy pictures were taken at time point 0 h and 24 h after the scratch and analysed. Data are expressed as area reduction from time points 0 h to 24 h as % of the Wt ctrl value and are means ± S.D. (n=4-6). **p<0.01 statistically significant compared to the Wt ctrl value. (C) Quiescent mouse mesangial cells isolated from either BalbC mice or $S1P_3$-deficient mice were stimulated for 10 min with either vehicle (Ctrl) or sphingosine 1-phosphate (S1P, 1 µM) in the presence of 1% FBS. Cell lysates were subjected to G-LISA® RhoA activation assay according to the manufacturer´s instructions. Data are expressed % of Wt ctrl value and are means ± S.D. (n=4). *p<0.05, ***p<0.001 considered statistically significant compared to the vehicle treated Wt value, §§§p<0.001 considered statistically significant compared to the S1P stimulated Wt value.

On the other hand, SK-2 was activated by epidermal growth factor (EGF) [24] due to ERK-mediated hyperphosphorylation in breast cancer cells. Such an activation of SK-2 by growth factors is hardly consistent with a growth inhibiting and pro-apoptotic effect of SK-2. In fact, we previously showed that in the breast cancer cell line MCF-7, EGF and estrogen rather down-regulated SK-2 mRNA expression [25,26] which occurred concomitantly to an up-regulation of SK-1 by these factors.

Recently, a catalytic inhibitor of SK-2 ABC294640 [27] was developed and used to address the cellular function of SK-2. Although the in vitro IC_{50} for SK-2 was rather high at 50 μM [14], oral administration of ABC294640 to mice bearing mammary adenocarcinoma xenografts resulted in a dose-dependent antitumor activity associated with depletion of S1P levels in the tumours and progressive tumour cell apoptosis [14,28]. These data suggested that SK-2 similar to SK-1 is also positively involved in cancer growth and progression. In our present study, treatment of mesangial cells with 30 and 100 μM ABC294640 resulted in a reduced proliferation rate, which however, was also detected in SK-2ko cells (Fig. 2C). These data clearly demonstrate that ABC294640 elicits an anti-proliferative effect independent of SK-2 inhibition, thereby questioning the specificity of ABC294640. Recently, ABC294640 was shown to exert anti-estrogenic effects by directly antagonising estrogen receptor (ER) activation [27]. Molecular modeling studies revealed a direct docking of ABC294640 into the ligand binding site of the ERα. This estrogen antagonistic potential may explain on the one hand the anti-proliferative effect and on the other hand why especially estrogen-sensitive breast cancer cells were strongly affected by the compound. The comparison of in vivo effects of ABC294640 with the effect of genetic SK-2 knockout mice also revealed inconsistent results. Whereas the inhibitor aggravated inflammatory arthritis, the SK-2 knockout mice showed no change of disease severity compared to wild-type mice [29]. Moreover, Liang et al. showed that

SK-2 deficient mice develop more severe chronic intestinal inflammation and colitis-associated cancer which in terms of mechanism, was proposed to involve a counter-upregulation of SK-1 and S1P [30].

Also our study showed a compensatory up-regulation of SK-1 protein in SK-2ko cells and SK-2-siRNA transfected cells. However, using LC-MS/MS we only detected a rise of cellular S1P in SK-2ko MEF but not in SK-2ko mMCs. The reason for this discrepancy might be due to a detection limit, since we only measured S1P in whole cell lipid extracts but not in subcellular fractions. Another possibility is that produced S1P is secreted more efficiently from mesangial cells than from fibroblasts. However, so far we are not able to measure an increased level in cell culture supernatants (data not shown). Thus, further experiments are needed to clarify this issue.

In addition, our data revealed that specifically the $S1P_3$ receptor was up-regulated upon loss of SK-2 (Fig. 7A and 7B). We therefore speculate that increased proliferation and migration observed in SK-2ko cells may be due to this enhanced $S1P_3$ expression. We obtained evidence that $S1P_3$ is involved in mesangial cell migration and proliferation from $S1P_3$ knockout mesangial cells as (i) the $S1P_3$ko cells showed a slower proliferation rate in comparison to wild-type cells (Fig. 9A) and (ii) S1P was able to induce cell migration only in wild-type but not in $S1P_3$ko cells (Fig. 9B). However, since mesangial cell proliferation was only partially reduced in $S1P_3$-deficient cells and also showed normal migration behaviour under control conditions, we conclude that other receptor subtypes may also be involved. Katsuma et al. reported that extracellular S1P triggers mesangial cell proliferation by involving both $S1P_2$ and $S1P_3$ [31]. Similarly, proliferation of hepatoma cells [32] and satellite cells [33] also depended on both $S1P_2$ and $S1P_3$. Concerning cell migration, we observed an increased migration of mesangial cells when inhibiting the $S1P_2$ receptor with JTE013, suggesting a potentially inhibitory role of $S1P_2$ in cell migration, which was previously also reported in other cell types [34–36].

Similar to $S1P_2$ also the involvement of $S1P_3$ in a migratory event is conflicting. In this respect, Okamoto et al. showed that an overexpression of $S1P_1$ and $S1P_3$ in Chinese hamster ovary (CHO) cells resulted in more S1P-stimulated migration [37]. A similar situation was reported for human endothelial cells where S1P-triggered migration was abolished by antisense oligonucleotides against $S1P_1$ and $S1P_3$ [38] and in lung cancer cells where either a $S1P_3$ knockdown by RNAi or Rho kinase inhibition by a catalytic inhibitor prevented a S1P-stimulated migratory/invasive response [39].

By generating S1P receptor knockout mice, it turned out that $S1P_1$ deficient mice are embryonically lethal since vascular maturation failed due to a defect in smooth muscle cells migration [40] and consistently, embryonic fibroblasts (MEF) isolated from these $S1P_1$ deficient mice also exhibited a defect in chemotaxis towards S1P [40]. These data clearly support an in vivo relevant function of $S1P_1$ in smooth muscle cell and fibroblast migration. In contrast, $S1P_3$ deficient mice showed no vascular maturation deficit which indirectly allowed the conclusion that $S1P_3$ is not involved in smooth muscle cell migration and consequently vessel maturation [40,41]. All these data do not allow a general conclusion, but somehow stress the possibility that $S1P_3$, eventually in cooperation with $S1P_1$, is involved in S1P-stimulated cell migration in a cell-type specific manner.

Our data further demonstrate that not only the basal but also the S1P-stimulated RhoA activity is enhanced in SK-2 deficient cells. RhoA is one of three members of the Rho GTPase family that is known to play a crucial role in the regulation of cell migration. There seems to be a spatiotemporal dynamic of RhoA action that drives not only membrane protrusions at the leading edge of the cells but also drives tail retraction [42]. Although previously regarded as an anti-migratory GTPase, the finding that RhoA localises at the leading edge of cells where it is important for membrane ruffling and lamellae formation supported a series of studies describing RhoA as pro-migratory factor [for

review see: 43]. As we were only able to measure RhoA activity in whole cell lysates, we cannot decipher the exact cellular localisation of the enhanced RhoA activity in SK-2ko cells to allow a final conclusion on the specific impact of RhoA acting pro-migratory on the leading edge or anti-migratory by tail retraction in mesangial cells. However, RhoA is activated by extracellular S1P in many cell types including CHO cells [37], endothelial cells [38], and bladder cancer cells [44], and this seems to be mandatory for cell migration. It is tempting to speculate that both $S1P_2$ and $S1P_3$ receptors may cooperatively orchestrate cell migration. All these data suggest that it is well conceivable that an enhanced SK-1/$S1P_3$/RhoA signalling may contribute to the increased migration in SK-2ko cells.

Notably, in SK-2ko cells there was a much more pronounced increase of sphingosine and C16-ceramide than of S1P (Fig. 7C, D) supporting speculations whether another sphingolipid mediator besides S1P may contribute to the observed effects in this study. In this regard, we and others have shown that ceramide can directly bind to and activate c-Raf [45], PKCα [46] and PKC-ζ [47] but inhibit PKC-δ [46,48]. Future studies are necessary to evaluate the exact contribution of the various sphingolipid-derived mediators to the observed phenotype of SK-2ko mesangial cells.

Although our study has only addressed the effect of SK-2ko on the cellular level on mesangial cells, it will be interesting to see whether in vivo in a renal disease model SK-2 deficient mice also respond with increased proliferation and migration. In this context, it was reported in a renal ischemia/reperfusion injury (IRI) model, that loss of SK-2 led to more severe kidney injury as indicated by elevated plasma creatinine levels, increased tubular cell necrosis, dilation of tubules, and cast formation in the outer medulla of SK-2ko mice. In addition, kidneys of IRI-exposed SK-2ko mice not only showed more neutrophil infiltration but remarkably also increased $S1P_3$

expression [49] which resembled our data obtained in renal mesangial cells and fibroblasts.

Altogether, these data show for the first time that a loss of SK-2 in renal mesangial cells and fibroblasts may have a considerable impact on cell growth and migration and that SK-2 exerts a suppressive effect on these cellular responses that are crucial in many renal diseases such as renal cancer and chronic inflammatory kidney diseases.

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Chapter 4

The ω3-polyunsaturated fatty acid derivatives AVX001 and AVX002 directly inhibit cytosolic phospholipase A_2 and suppress PGE_2 formation in mesangial cells

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British Journal of Pharmacology (2012) 167 (8): 1691 – 701

In this project I participated in studying of effectiveness of AVX001 and AVX002 compounds for reduction of cytokine-stimulated PGE_2 formation in mesangial cells (Fig. 4).

Abstract

Omega3-polyunsaturated fatty acids (ω3-PUFA) are known to exert anti-inflammatory effects in various disease models although the direct targets are only poorly characterized. Here we report on two new cPLA$_2$ inhibitors, the ω 3-derivatives 1-octadeca-2,6,9,12,15-pentaenylsulfanyl-propan-2-one (AVX001) and 1-octadeca-3,6,9,12,15-pentaenylsulfanyl-propan-2-one (AVX002) and their effects on inflammatory prostaglandin E_2 (PGE_2) production in cultures of renal mesangial cells. AVX001 and AVX002 dose-dependently inhibited the group IVA cytosolic phospholipaseA_2 (cPLA$_2$) in an in vitro activity assay with a calculated IC_{50} value of 112 nM for AVX001 and 131 nM for AVX002 whereas the known cPLA$_2$ inhibitor arachidonoyl trifluoromethyl ketone ($AACOCF_3$) was less potent and docosahexaenoic acid (DHA) was inactive. In renal mesangial cells, AVX001 and AVX002 suppressed interleukin-1β (IL-1β)-induced PGE_2 synthesis. Mechanistically, this effect occurred by a downregulation of IL-1β-induced group IIA-sPLA$_2$ protein expression, mRNA expression and promoter activity. A similar but less potent effect was seen with $AACOCF_3$ and no effect was seen with DHA. Since gene expression of sPLA$_2$ is known to be regulated by the transcription factor nuclear factor κB (NFκB), we further investigated NFκB activation. Both compounds prevented NFκB activation by blocking degradation of the inhibitor of κB (IκB). These data show for the first time that the novel cPLA$_2$ inhibitors AVX001 and AVX002 exerted an anti-inflammatory potential in cultures of renal mesangial cells and reduced the pro-inflammatory mediator PGE_2 through an inhibitory effect on NFκB activation. Therefore, these compounds may represent promising novel drugs for the treatment of inflammatory disorders.

4.1. Introduction

Mesangial cells are specialized smooth muscle-like cells located in the renal glomerulus and are not only involved in the regulation of the glomerular filtration rate and in the preservation of the structural integrity of the glomerulus, but also play a central role in most pathological processes of the renal glomerulus [1,2]. Upon activation by a variety of pro-inflammatory cytokines, mesangial cells respond with three prominent reactions which are all hallmarks of many forms of glomerulonephritis: (i) increased proliferation, (ii) increased mediator production including cytokines and chemokines, nitric oxide[1] (NO) and superoxide radicals, and prostaglandins (PG), and (iii) increased extracellular matrix production [1]. The detailed mechanisms underlying these cell responses are still not completely understood.

One important event in the inflammatory reaction and the rate-limiting step in the generation of prostaglandins is the activation of a phospholipase A_2 (PLA_2) which hydrolyzes the *sn*-2 ester bond of substrate phospholipids and thereby generates arachidonic acid and lysophospholipids [3]. Arachidonic acid is then further converted by either cyclooxygenases or lipoxygenases and downstream enzymes to the eicosanoids including prostaglandins, thromboxanes and leukotrienes.

So far, 15 groups and many subgroups of PLA_2s have been identified which include five distinct types of enzymes, i.e. secreted PLA_2s ($sPLA_2$), the cytosolic PLA_2s ($cPLA_2$), the Ca^{2+}-independent PLA_2s ($iPLA_2$), the platelet-activating factor acetylhydrolases (PAF-AH), and the lysosomal PLA_2s [3].

Since $cPLA_2$ preferentially hydrolyses arachidonic acid-containing phospholipids at the *sn*-2 position, this enzyme is thought to be one key enzyme in inflammatory eicosanoid formation.

In renal mesangial cells, four PLA_2 subtypes are expressed either constitutively or inducibly. These include the $cPLA_2$, the $iPLA_2$, and the group

IIA and V sPLA$_2$ [4–7]. In previous studies on mesangial cells, it was shown that the drastic increase of cytokine-triggered PGE$_2$ formation involved both IIA-sPLA$_2$ and cPLA$_2$ activation [8]. By using the specific sPLA$_2$ inhibitor CGP43187 or by using a neutralizing antibody against IIA-sPLA$_2$, about 80 % of the cytokine-triggered PGE$_2$ formation was depleted suggesting that the remaining small amount of approx. 20 % of PGE$_2$ derives from cytokine-stimulated cPLA$_2$ activity [8]. Indeed, the cPLA$_2$ is not only acutely activated by interleukin 1 (IL-1) [5], but also upregulated upon prolonged IL-1 treatment by a transcriptional mechanism [4,9]. Furthermore, a cross-communication exists between the different PLA$_2$s. Thus, in mesangial cells, we previously showed that the IIA-sPLA$_2$, acting from the outside of cells, is able to activate the cPLA$_2$ intracellularly via a protein kinase C (PKC) and mitogen activated protein kinase (MAPK) dependent mechanism [10]. In mouse P388D1 macrophages, it was shown that cPLA$_2$ also contributes to V-sPLA$_2$ activation [11]. All these data suggest that the different PLA$_2$s regulate each other and are critically participating in pro-inflammatory PGE$_2$ formation.

In this study we have identified and characterized two novel direct cPLA$_2$ inhibitors, i.e. the ω3-PUFA derivatives AVX001 and AVX002. We show that in cultures of renal mesangial cells, AVX001 and AVX002 downregulated cytokine-stimulated PGE$_2$ formation through a mechanism blocking cytokine-triggered and cPLA$_2$-dependent NFκB activation and subsequent gene transcription of sPLA$_2$. These data suggest that AVX001 and AVX002 could serve as novel anti-inflammatory drugs.

4.2. Materials and methods

4.2.1. AVX001 and AVX002

Both compounds were synthesized and characterized according to Holmeide and Skattebol (2000) [12] and kindly provided by Dr. Inger Reidun Aukrust and Dr. Marcel Sandberg (Synthetica AS, Norway). The chemical structures are indicated in Fig. 1.

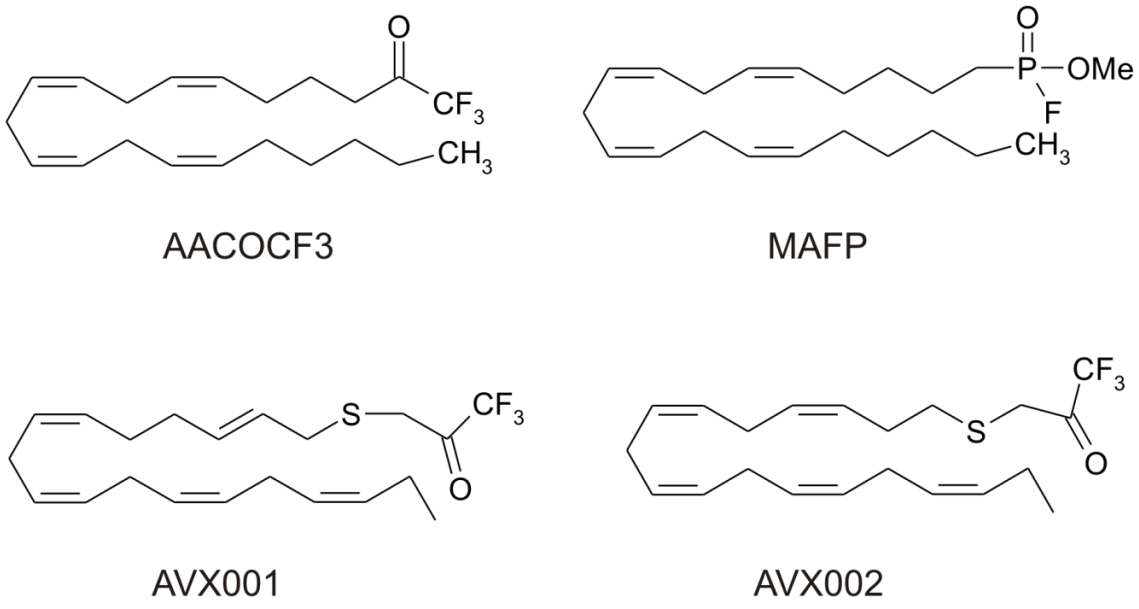


Fig. 1. Chemical structure of various $cPLA_2$ inhibitors.

Both compounds were analyzed by HPLC for purity (Fig. 2). For this, all peaks in the chromatogram were taken for area integration using an integrated HPLC software. The purities were determined as 97 % for AVX001 and 92.7 % for AVX002. Both compounds were stored at -80°C at a 20 mM stock solution in DMSO under argon gas to minimize oxidation.

4.2.2. Cell culture

Rat renal mesangial cells were isolated, characterized, and cultured as previously described [13]. For the experiments performed in this study, cells between passages 8-30 were used.

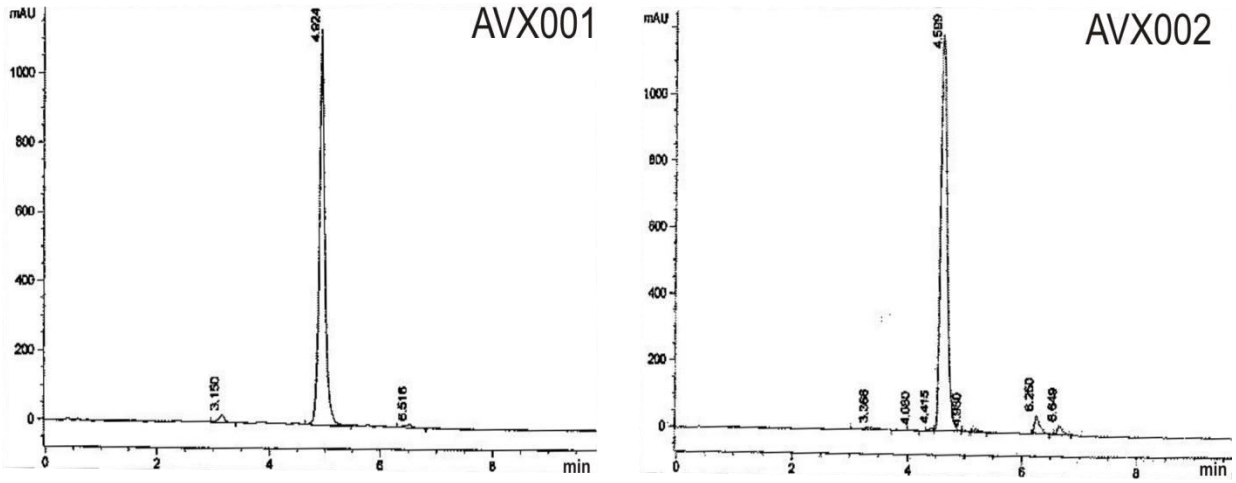


Fig. 2. HPLC profiles of AVX001 and AVX002. The peak at 4.924 min (left panel) corresponds to AVX001. The peak at 4.599 min (right panel) corresponds to AVX002.

4.2.3. In vitro $cPLA_2$ activity assay

Recombinant human $cPLA_2$ enzyme was used for an in vitro activity assay as described by Wijkander et al [14] with some modifications according to Lucas and Dennis [15]. Enzyme with inhibitor (in DMSO, final concentration 1 %) or solvent alone was preincubated in assay buffer (1 mM EDTA, 80 mM KCl and 10 mM HEPES (pH 7.4) containing 1.56 mM $CaCl_2$, and 2.36 mM dithiothreitol for 80 sec at 37°C and 10 min at 23°C. Lipid vesicles were prepared by drying 4.2 nmol of L-α-1-palmitoyl-2-arachidonyl-[arachidonyl-1-^{14}C]-phosphatidylcholine under a stream of nitrogen. Dried lipids were resuspended in 2 ml assay buffer and sonicated twice for 7 min (setting: output 3.5 and 50 % duty cycles) in a Branson Sonifier 250 (Branson Ultrasonic

Corporation, Danbury, CT). Sonicated lipid vesicles were added to the reaction to a final concentration of 0.2 μM. The reaction mixture was incubated for one hour at 37°C and stopped by addition of 1.7 ml chloroform/methanol/37% KCl/ 0.45M BHT/ 0.33M AA (2:1:0.01:0.015:0.005, by vol). After phase separation the lower phase was transferred to a glass tube, dried under nitrogen, and resuspended in chloroform/methanol (9:1, vol vol^{-1}) and applied to a silicagel thin layer chromatography. Free [1-^{14}C] arachidonic acid and L-β-1-palmitoyl-2-arachidonyl-[arachidonyl-1-^{14}C] phosphatidylcholine (Perkin Elmer) were separated and analysed as described by Anthonsen et al [16].

4.2.4. PGE_2 determination

Confluent mesangial cells in 24 well plates were pretreated for 90 min in the presence or absence of the inhibitors prior to stimulation for 24 h in a volume of 0.5 ml with IL-1β (1 nM) to induce PGE_2 formation. Thereafter, equal volumes of supernatants were subjected to a PGE_2-ELISA according to the manufacturer's instructions. Data were calculated as pg of PGE_2 per 1.3 x 10^5 cells which was the cell number per well.

4.2.5. Cell stimulation and Western blot analysis

Confluent mesangial cells were stimulated as indicated in the figure legends in Dulbecco's modified Eagle medium (DMEM) supplemented with 0.1 mg ml^{-1} of fatty acid-free bovine serum albumin and 10 mM HEPES. After stimulation, the supernatant was taken for detection of secreted IIA-$sPLA_2$. The cell monolayers were homogenized in lysis buffer and processed exactly as previously described [17]. Cell lysates were taken for protein determination. Lysates containing 60 μg of protein, were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and subjected for Western blot analysis using antibodies as

indicated in the figure legends. Bands were detected by enhanced chemiluminescence method as recommended by the manufacturer.

4.2.6. Detection of secreted IIA-$sPLA_2$

Equal volumes of supernatants derived from the same number of cells were taken for protein precipitation using 7 % (w vol^{-1}) of trichloroacetic acid. Precipitated proteins were redissolved in SDS-Laemmli buffer without dithiothreitol and subjected to SDS-PAGE (15 % acrylamide gel), transferred to nitrocellulose membranes and immunostained by using a monoclonal antibody against rat IIA-$sPLA_2$ at a dilution of 1:60 in 0.01 % milk powder containing PBS as previously described [18]. GAPDH was stained and densitometrically evaluated in the corresponding cell lysates and used for normalization by calculating the ratio between secreted $sPLA_2$ and cytosolic GAPDH for each sample.

4.2.7. Quantitative PCR analysis

2 µg of total RNA were used for reverse transcriptase (RT)-PCR (first strand cDNA synthesis kit, MBI Fermentas, St.Leon-Rot, Germany) using random hexamer primers for amplification. mRNA levels were determined by quantitative real-time PCR. The following primers were used: rat $sPLA_2$-IIA: forward: GCC AAA TCT CCT GCT CTA CAA ACC, reverse: ACT GGG CGT CTT CCC TTT GC; 18S RNA (forward: CGA TTC CGT GGG TGG TGG TG, reverse: CAT GCC AGA GTC TCG TTC GTT ATC); iQ™ SYBR® Green Supermix was from BIORAD. The cycling conditions were as following: initial activation step (95°C/3 min), followed by 40 cycles of denaturation (95°C/15 sec) and annealing (58°C/1 min). PCR products were detected by monitoring the increase in fluorescence with iCycler, iQ™5 Multicolor Real-Time PCR Detection System, BIORAD. The BIORAD iQ5 Standard Edition

Optical System Software Version 2.0 was used to analyze real time and end point fluorescence.

4.2.8. Cell transfection and luciferase reporter gene assay

A 2.67 kb fragment of the rat $sPLA_2$-IIA promoter was cloned according to a previous report and fused to a luciferase reporter gene [19]. Cells were cultured in 12-well plates and transfected with 0.3 µg of the promoter-containing plasmid plus 0.03 µg of a plasmid containing the renilla luciferase gene by using Effectene Reagent (QIAGEN, Germany) according to the manufacturer's instructions. Thereafter, the transfection medium was removed and cells were stimulated as indicated. Values for the relative gene promoter activities were calculated from the ratio of firefly / renilla luciferase activities.

4.2.9. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test for multiple comparisons (GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, USA).

4.2.10. Chemicals

IL-1β was from Cell Concept GmbH, Umkirch, Deutschland; hyperfilm MP and horseradish-coupled secondary antibodies were from GE Healthcare Systems, Freiburg, Germany; $AACOCF_3$, DHA, and the PPARγ antagonist G3335 were from Merck Biosciences, Schwalbach, Germany; the monoclonal antibody against rat IIA-$sPLA_2$ was generated and characterized as previously described [20]; the IκB-α antibody was from Cell Signalling, Frankfurt am Main, Germany; the PGE_2 enzyme-linked immunosorbent assay (ELISA) was from Assay Designs, BIOTREND Chemikalien GmbH, Köln, Germany; all cell culture nutrients were from Invitrogen/Life Technologies, Karlsruhe, Germany.

4.3. Results

4.3.1. AVX001 and AVX002 inhibit in vitro $cPLA_2$ activity and reduce cytokine-stimulated PGE_2 formation in rat renal mesangial cells

Previously, it was shown that the ω6-PUFA derivatives arachidonyl-trifluoromethyl ketone ($AACOCF_3$, ATK; Fig. 1) [21] and methyl-arachidonyl fluorophosphonate (MAFP; Fig. 1) [22] are direct inhibitors of the group IVA $cPLA_2$ in vitro. In the present study, we tested the ability of AVX001 and AVX002 to inhibit $cPLA_2$ activity. In these compounds the methylene group β to the carbonyl group of the ketone in $AACOCF_3$ was replaced by a sulphur atom. Holmeide and Skattebol speculated that this would make the carbonyl carbon more electrophilic and consequently the molecule a more potent inhibitor of $cPLA_2$ [12]. The purity of these compounds were analyzed by HPLC separation (Fig. 2) and determined to be 97 % for AVX001 and 92.7 % for AVX002. *In vitro* activity assays revealed that AVX001 blocked $cPLA_2$ activity in a concentration-dependent manner (Fig. 3). Multiple experiments were taken for the determination of the IC_{50} value which was calculated as 120 ± 58 nM (n=28). The structurally similar compound AVX002 also blocked $cPLA_2$ activity in vitro (Fig. 3). The IC_{50} value was analysed from several experiments to be 126 ± 37 nM (n=14). By comparison, $AACOCF_3$ was a less potent inhibitor; it showed 30 % inhibition at 0.3 μM and 72 % inhibition at 1μM. In contrast, the ω3-fatty acid docosahexaenoic acid (DHA) had no direct inhibitory effect on $cPLA_2$ (Fig. 3). These data suggest that not only ω6-PUFA derivatives, but also certain ω3-PUFA derivatives, such as AVX001 and AVX002, but not DHA, are effective direct $cPLA_2$ inhibitors.

To further test the effectiveness of AVX001 and AVX002 in a cell culture system, rat renal mesangial cells were used which represent a good model system to investigate molecular inflammatory mechanisms [2]. Treatment of

mesangial cells with IL-1β led to a high production of PGE_2 confirming many previous reports of our group [8,23,24].

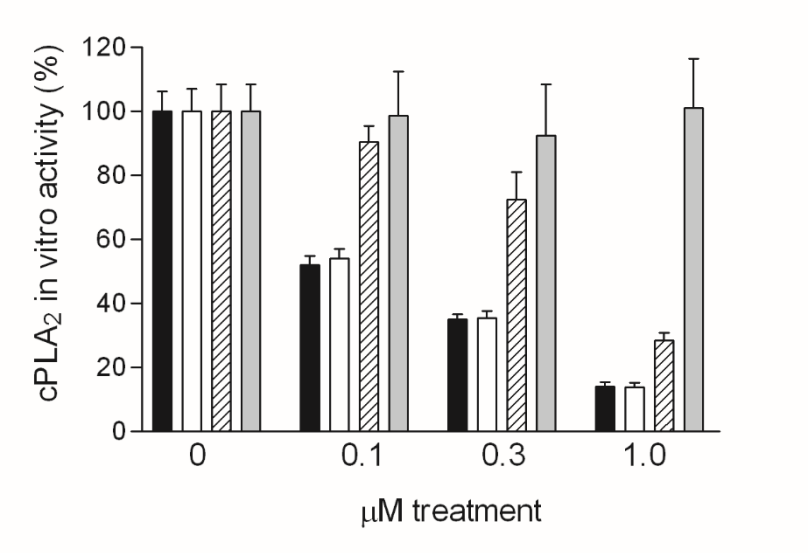


Fig.3: Direct inhibiting effect of AVX001, AVX002, ATK and DHA on $cPLA_2$ activity in vitro. Recombinant $cPLA_2$ enzyme was incubated with either solvent (DMSO, final concentration 1 %, 0) or the indicated concentrations of AVX001 (closed bars) or AVX002 (open bars), $AACOCF_3$ (hatched bars), and DHA (dotted bars) and taken for an in vitro activity assay as described in the Methods Section. $cPLA_2$ enzyme activity is given as % of control value (activity in the absence of inhibitors). The results shown are representative of at least three independent experiments, and data represent mean of duplicate determinations.

The average amount of PGE_2 in different experiments varied from 0-55 pg per 1.3 x 10^5 cells in unstimulated cells, to 2359-3751 pg per 1.3 x 10^5 cells in IL-1β-stimulated cells. Due to this variability which seemed to depend on the cell passages, the maximal IL-1β-stimulated values were always set to 100 %. In the presence of AVX001, PGE_2 formation was concentration-dependently reduced with a 90 % inhibition at 10 μM of concentration (Fig. 4). A similar inhibitory effect was also seen for AVX002 (Fig. 4). $AACOCF_3$ also reduced PGE_2 levels but was less potent, and docosahexaenoic acid (DHA) even at 20 μM had no significant effect (Fig. 4).

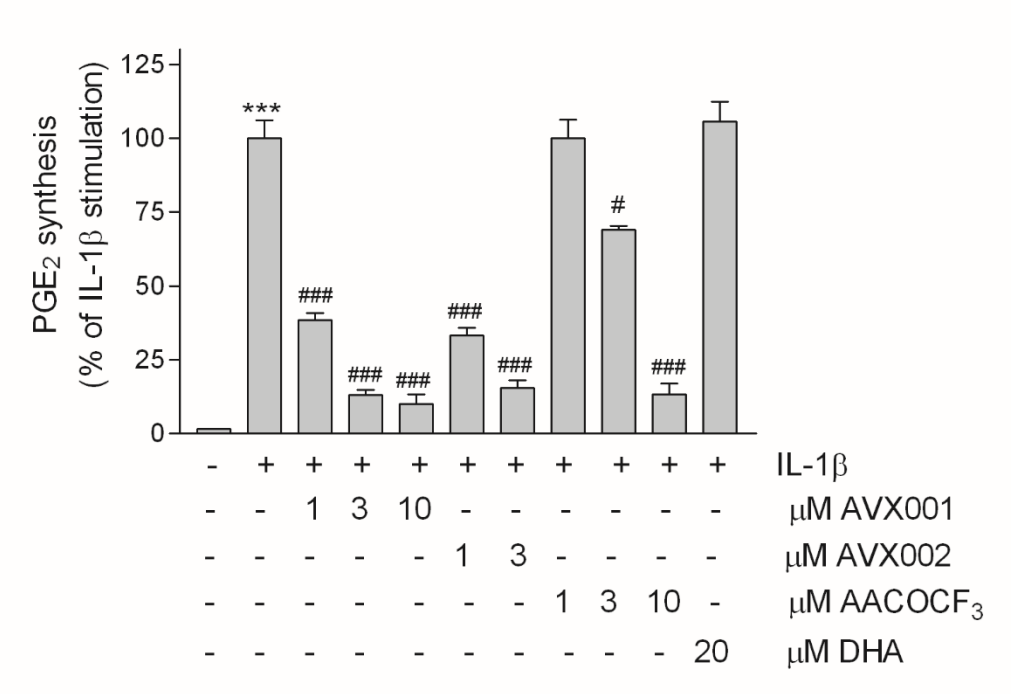


Fig. 4: Effect of AVX001 and AVX002 on IL-1β-stimulated PGE_2 formation in mesangial cells. Quiescent cells were stimulated for 24 h with either DMEM (-), and IL-1β (1 nM) in the absence (-) or presence of the indicated concentrations (in μM) of AVX001, AVX002, $AACOCF_3$, and docosahexaenoic acid (DHA). The inhibitors were all pretreated for 90 min prior to stimulation. Supernatants were collected and taken for PGE_2 quantification by using an ELISA. Data are expressed as % of maximal IL-1β-stimulated PGE_2 and are means ± S.D. (n=3).***$p<0.001$ considered statistically significant when compared to the control values; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ when compared to the IL-1β-stimulated values.

Since we previously showed that the cytokine-induced PGE_2 formation in mesangial cells involves both $sPLA_2$ and $cPLA_2$ activation [8], we then investigated the effect of AVX001 on $sPLA_2$ protein and mRNA expressions. As seen in Fig. 5A, AVX001 and AVX002 both concentration-dependently down-regulated the $sPLA_2$ protein expression paralleling the reduced PGE_2 formation. To verify that supernatants derived from equal cell amounts, corresponding cell monolayers were lysed and stained for GAPDH protein expression (Fig. 5A, inset, lower panels) and used for normalization of secreted proteins. A similar reducing effect on $sPLA_2$ protein expression was also seen

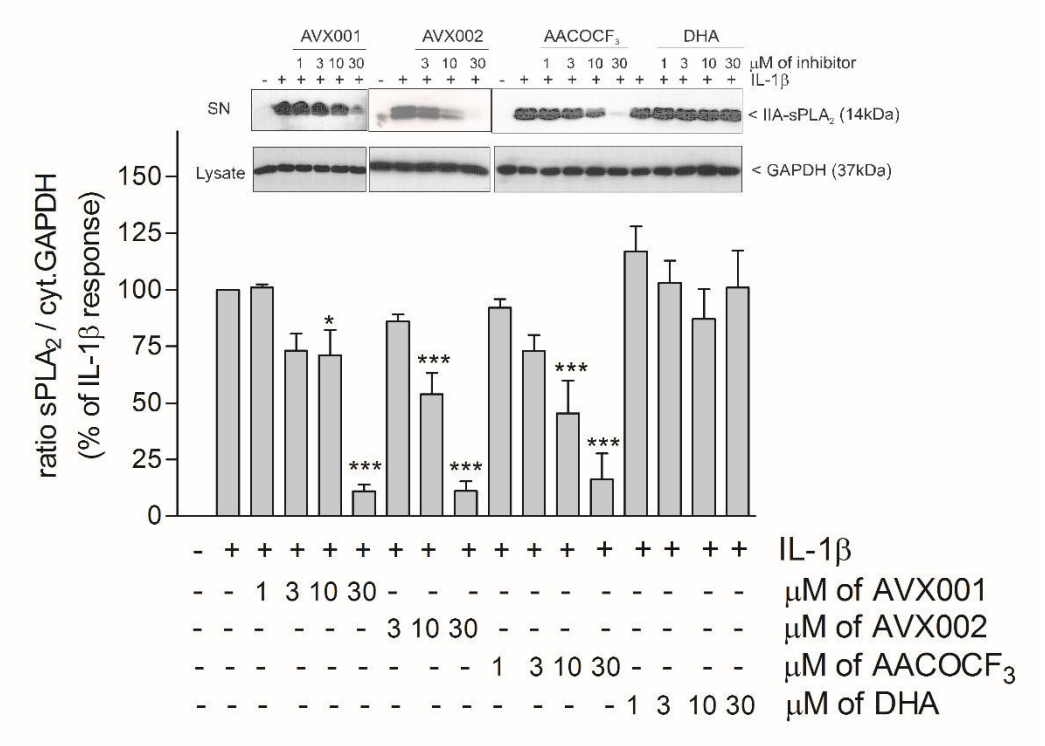
for $AACOCF_3$, whereas DHA had no significant effect (Fig. 5A). IIA-$sPLA_2$ mRNA expression was also reduced by AVX001 and AVX002 (Fig. 5B). Again, $AACOCF_3$ was less potent and DHA was ineffective in reducing IIA-$sPLA_2$ mRNA expression (Fig. 5B). Notably, higher concentrations of AVX001 and AVX002 were needed to down-regulate the $sPLA_2$ protein and mRNA expressions compared to the reduction of PGE_2.

To see whether this effect on $sPLA_2$ protein and mRNA expression was due to a reducing effect on $sPLA_2$ promoter activation and subsequent gene transcription, luciferase reporter gene assays were performed. A 2.26 kb fragment of the rat IIA-$sPLA_2$ promoter was cloned according to Scholz-Pedretti et al. (2002) and ligated into a luciferase-containing vector (pGL3) and used to transfect mesangial cells [19]. AVX001 and AVX002 at 20 μM significantly reduced IL-1β-stimulated promoter activity (Fig. 5C) suggesting that $cPLA_2$-regulated transcription factors are essential for $sPLA_2$ gene transcription.

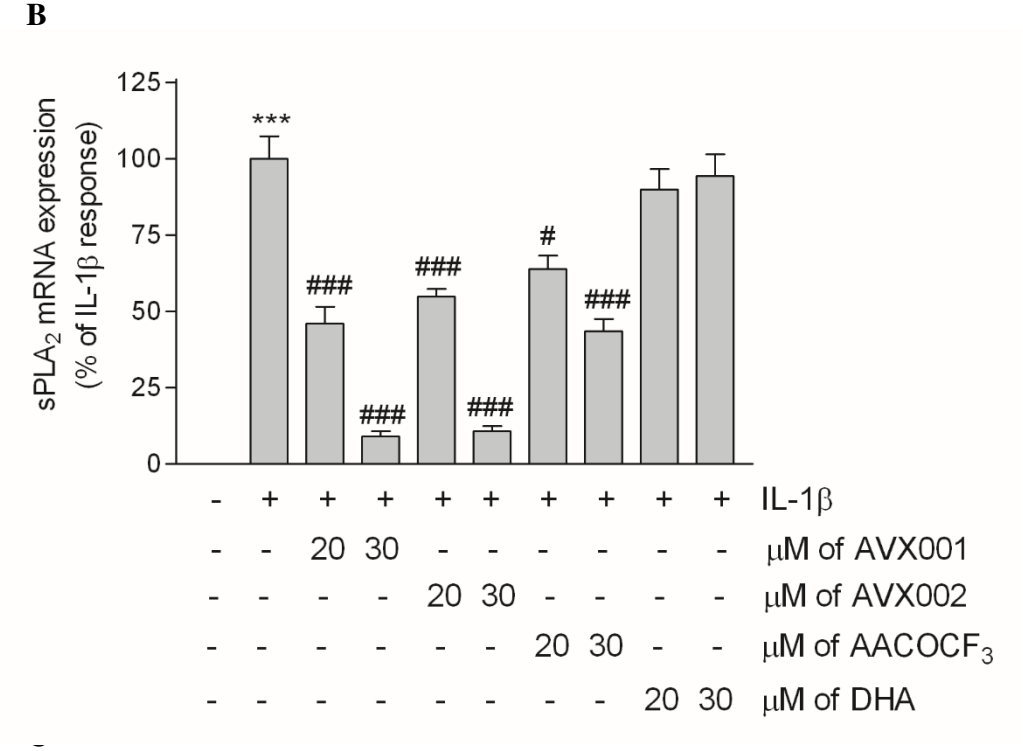
4.3.2. AVX inhibitors block cytokine-stimulated NFκB activity in mesangial cells.

Since it is well known that the transcription factor NFκB is crucially involved in $sPLA_2$ gene transcription under inflammatory conditions [25] we further studied whether the AVX inhibitors had an effect on NFκB activation. NFκB activation was measured indirectly by Western blot analysis of the inhibitor of κB (IκB) protein expression. Short-term stimulation of cells for 15 min with IL-1β revealed a pronounced degradation of IκB protein (Fig. 6) which is a key event in NFκB activation that releases NFκB from its sequestration in the cytoplasm and allows its nuclear translocation. This downregulating effect of IL-1β on IκB was reverted by the IκB inhibitor Bay11-7085, but also by AVX001 and AVX002 (Fig. 6).

A


AVX001
AVX002
AACOCF3
DHA
μM of inhibitor
IL-1β
SN
< IIA-sPLA2 (14kDa)
Lysate
< GAPDH (37kDa)
ratio sPLA2 / cyt.GAPDH
(% of IL-1β response)
IL-1β
μM of AVX001
μM of AVX002
μM of AACOCF3
μM of DHA

B


sPLA2 mRNA expression
(% of IL-1β response)
IL-1β
μM of AVX001
μM of AVX002
μM of AACOCF3
μM of DHA

C

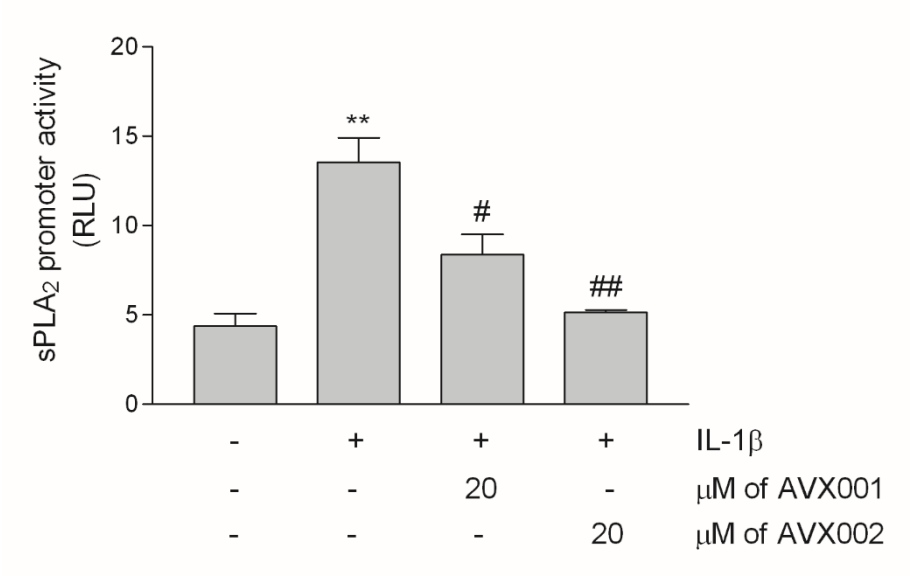


Fig. 5: Effect of AVX001 and AVX002 on IL-1β-stimulated sPLA$_2$ protein (A), mRNA expression (B), and promoter activity (C) in mesangial cells. Quiescent cells were stimulated with either DMEM (-), or IL-1β (1 nM) in the absence (-) or presence of the indicated concentrations of AVX001, AVX002, AACOCF$_3$, and DHA (all inhibitors were pretreated for 90 min prior to stimulation. (A) Supernatants were taken for protein precipitation, separation by SDS-PAGE, transfer to membranes, and Western blot analysis using a monoclonal antibody against rat sPLA$_2$. Corresponding cell lysates were stained for GAPDH and used for normalization. Bands were densitometrically evaluated and the ratio between secreted sPLA$_2$ and cytosolic GAPDH was calculated. Results are expressed as % of IL-1β stimulation. Data are means ± S.D. (n=3-5). The inset shows representative Western blots of sPLA$_2$ (upper panels) from supernatants (SN) and GAPDH (lower panels) from cell lysates. (B) Cells were taken for RNA extraction and subjected to quantitative PCR analysis of rat IIA-sPLA$_2$ and 18S RNA. Δ Δ Ct values were calculated and results are expressed as % of maximal IL-1β-stimulated response and are means ± S.D. (n=3-5). (C) Cells were transfected with the sPLA$_2$ promoter construct plus a plasmid coding for the Renilla luciferase. After transfection, cells were stimulated for 24 h with vehicle (-), IL-1β (1 nM), or IL-1β in the absence or presence of AVX001 or AVX002 (both at 20 μM). Both inhibitors were preincubated for 90 min prior to stimulation. sPLA$_2$ promoter activity was calculated and results are expressed as relative luciferase units (RLU) and are means ± S.D. (n=3). **p<0.01, ***p<0.001 considered statistically significant when compared to the control values; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 when compared to the IL-1β-stimulated values.

4.3.3. AVX001 and AVX002 do not act through PPARγ activation.

Since AVX001 and AVX002 are structurally derived from ω3-fatty acids, and it has been reported previously that ω3-polyunsaturated fatty acids are ligands and activators of peroxisome proliferator-activated receptors (PPAR) [26], which have been attributed an anti-inflammatory effect [27], we further tested whether the reducing effect of AVX compounds on PGE_2 are mediated through PPARγ activation. Indeed, we found that in the presence of the PPARγ antagonist G3335, the AVX001 and AVX002 effects are partially reverted (Fig. 7). However, also the IL-1β effect was increased to the same extent in the presence of G3335 (Fig. 7) supporting the conclusion that there is rather a general stimulating effect of the PPARγ antagonist on the PGE_2 pathway than a specific antagonistic effect on the AVX action.

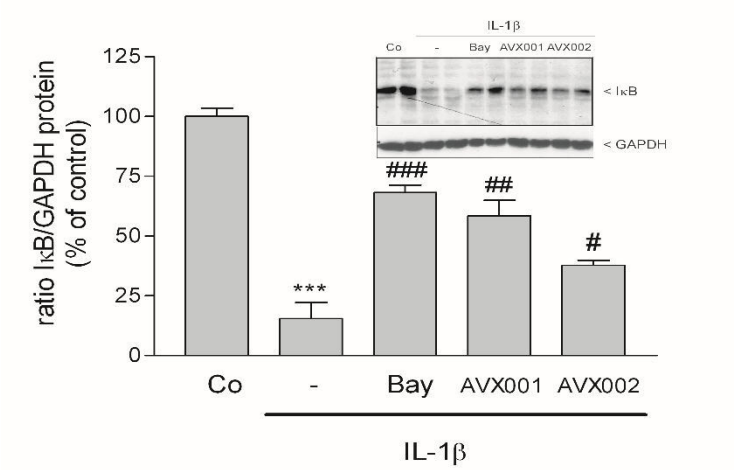


Fig. 6: Effect of AVX inhibitors on IL-1β-stimulated NFκB activation in mesangial cells. Quiescent cells were stimulated for 15min with either vehicle (Co), or IL-1β (1 nM) in the absence (-) or presence of Bay11-7085 (Bay, 10 μM), AVX001 or AVX002 (both at 30 μM). Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subjected to a Western blot analysis using a polyclonal antibody against IκBα (inset, upper panel) and GAPDH (inset, lower panel). Bands corresponding to IκB and GAPDH were densitometrically evaluated and the ratio between IκB and GAPDH was calculated for each sample. Results are depicted as % of control stimulations and are means ± S.D. (n=3). ***$p<0.001$ considered statistically significant when compared to the control values; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ when compared to the IL-1β-stimulated values. The inset shows a representative experiment in duplicates.

4.4. Discussion

In the last years, ω3-PUFAs have attracted a lot of interest due to accumulating evidence for their beneficial effects in various inflammatory diseases. These include rheumatoid arthritis, asthma, colitis, atherosclerosis, neurodegenerative diseases, and even certain forms of cancer [28–30].

Various possibilities for the mechanism of these anti-inflammatory effects were forwarded. For example, an increased incorporation of ω3-PUFA species into phospholipids may occur at the expense of arachidonic acid. Consequently, they replace arachidonic acid as a substrate for cyclooxygenases and lipoxygenases resulting in reduced formation of PGE_2 [31], TXA_2 [32], LTB_4 and LTE_4 [33]. Instead, an increased generation of other less active prostaglandin and leukotriene subspecies occurs [31,32,34]. Furthermore, it was proposed that ω3-PUFA may change the membrane lipid composition and thereby directly affect the functions of immune cells including the phagocytotic activity of macrophages, T cell signalling and proliferation, and antigen presentation activity of dendritic cells [35–37].

Various previous studies have shown that ω3-PUFAs are able to down-regulate the gene transcription of pro-inflammatory and growth promoting genes by interfering with transcription factors. In this view, Liu et al. showed that in a mouse epidermal cell system, ω3-PUFAs, but not the ω6-arachidonic acid, efficiently inhibited growth factor-triggered activator protein-1 transactivation [38]. Moreover, ω3-PUFAs downregulated cytokine-triggered NFκB activation and subsequent gene transcription in endothelial cells [39] as well as in macrophages [40]. However, in none of these studies the direct PUFA target was addressed.

Intriguingly, it was recently shown that ω3-PUFAs can act as ligands of peroxisome proliferator-activated receptors (PPARs) and thereby can modulate a multitude of pro- and anti-inflammatory genes [26,41,42]. In fact, the

appreciated lipid-lowering effect of fish oil supplementation [43] is mainly explained by the action of ω3-PUFAs or metabolites as PPAR agonists [41,44]. ω3-PUFAs can also act as ligands for the orphan receptor GPR120 and it was shown that this action contributes to anti-inflammatory and insulin-sensitizing effects in macrophages [45].

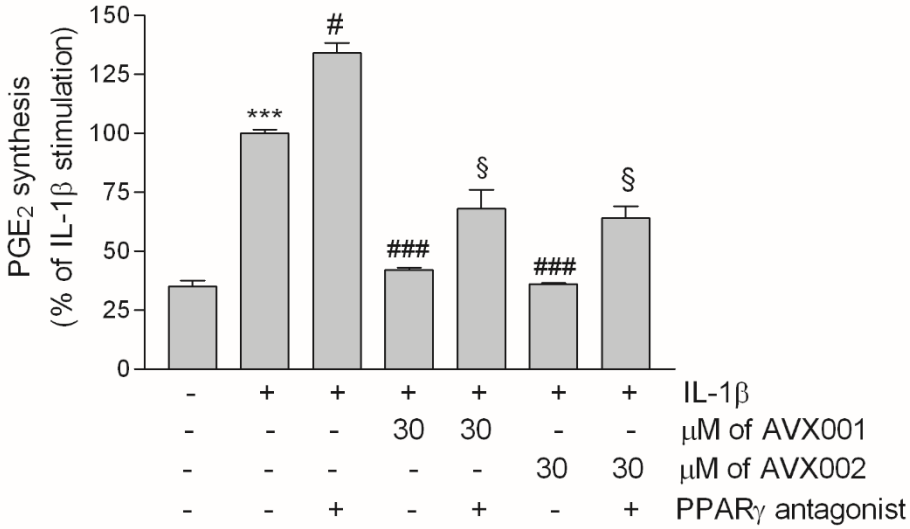


Fig. 7: Effect of the PPARγ antagonist G3335 on AVX001 and AVX002 reduced PGE$_2$ formation in mesangial cells. Quiescent cells were stimulated for 24 h with either DMEM (-), and IL-1β (1 nM) in the absence (-) or presence of 30 μM AVX001 or AVX002, in the absence or presence of 10 μM of G3335. The inhibitors were all pretreated for 90 min prior to stimulation. Supernatants were collected and taken for PGE$_2$ quantification by using an ELISA. Data are expressed as % of maximal IL-1β-stimulated PGE$_2$ and are means ± S.D. (n=3).***$p<0.001$ considered statistically significant when compared to the control values; $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ when compared to the IL-1β-stimulated values; $^{\S}p<0.05$ when compared to the corresponding AVX001 or AVX002 values.

Our data now clearly show that certain ω3-PUFA derivatives, such as AVX001 and AVX002, can also directly inhibit cPLA$_2$ in vitro as well as in intact cells. As a consequence, they are able to downregulate cytokine-stimulated PGE$_2$ formation in mesangial cells and thereby reduce an

inflammatory reaction. Mechanistically, we could further show that cPLA$_2$ is involved in crossregulation of sPLA$_2$-IIA and iNOS expressions via NFκB activation. This is stressed by our findings that the direct cPLA$_2$ inhibitors AVX001 and AVX002 not only down-regulated cytokine-induced sPLA$_2$ promoter activities (Fig. 5C), mRNA expression (Fig. 5B), protein expression (Fig. 5A) and subsequent activity (Fig. 4), but also, as a more upstream event, blocked IκB degradation and subsequent NFκB activation (Fig. 6). Notably, higher concentrations of AVX001 and AVX002 were needed to down-regulate the sPLA$_2$ protein and mRNA expressions compared to the reduction of PGE$_2$. The more potent effect on PGE$_2$ formation than on sPLA$_2$ expression may be derived from the dual action of AVX compounds, i.e. the direct inhibition of cPLA$_2$ (this enzyme accounts for approx. 20% of the cytokine-induced PGE$_2$ formation according to Pfeilschifter et al. [8]), and the partial down-regulation of sPLA$_2$ expression (this enzyme accounts for the remaining 80% of cytokine-induced PGE$_2$ formation according to Pfeilschifter et al. [8]).

It is also worth noting that we previously reported that in the human keratinocyte cell line HaCaT, three previously known cPLA$_2$ inhibitors, i.e. AACOCF$_3$, MAFP, and the trifluoromethyl ketone analogue of EPA (EPACOCF$_3$) all inhibited TNFα-induced NFκB activation and ICAM-1 expression [46]. The reduced NFκB activity could be reversed by addition of an excess of exogenous arachidonic acid [16], which further underlined the involvement of cPLA$_2$ in NFκB activation. Additional evidence that cPLA$_2$ is positively regulating NFκB activation was also presented by Camandola et al. [47]. These authors showed that in promonocytic U937 cells, arachidonic acid stimulated NFκB activation by a mechanism involving a metabolization of arachidonic acid to PGs and LTs.

However, when considering the fact that ω3-PUFAs can act as PPAR agonists, and the fact that the IIA-sPLA$_2$ gene contains functional PPAR binding elements (PPRE) in its promoter sequence [19], and in view of the

recent report that parts of the anti-inflammatory effect of PPARs may be mediated by direct negative interference with transcription factors such as NFκB, AP-1 and C/EBP [48], it may have been possible that the observed reducing effect of the AVX compounds on PGE_2 formation reported in this study also involves additional mechanisms besides the direct inhibition of $cPLA_2$ such as PPAR activation. We have therefore used a PPARγ antagonist to see whether the AVX001- and AVX002-mediated effects on PGE_2 could be reversed. Indeed, the down-regulated PGE_2 formation was increased in the presence of a PPARγ antagonist (Fig. 7). However, since also the IL-1β-triggered PGE_2 formation is increased by the PPARγ antagonist, we concluded that there is more a general PGE_2 stimulating effect by PPARγ antagonism than a specific effect on AVX action.

PLA_2 activation is the rate-limiting step in the generation of eicosanoids. It is also the initial step in the generation of PAF which is a further potent inflammatory mediator. Therefore, the pharmacological inhibition of PLA_2 is considered an attractive target to block inflammatory processes and should, at least theoretically, be a more effective approach than blocking one of the downstream enzymes like the cyclooxygenases. Unfortunately, the development of PLA_2 inhibitors has been hampered by the fact that too many subtypes of PLA_2 exist that are involved in a redundant manner in the inflammatory reaction [49–51].

Very few PLA_2 inhibitors have been developed which also exerted in vivo efficacy in various inflammatory animal models (reviewed in Huwiler and Pfeilschifter [52]). These include an inhibitor of IIA-$sPLA_2$, LY311727 [53], which proved efficient in a rat model of inflammatory pain [54]. The synthesis of novel IIA-$sPLA_2$ inhibitors has recently been described, which also exerted in vivo activity and reduced carrageenan-induced edema formation in rats with a similar potency as indomethacin at the dose of 10 mg kg^{-1} [55].

Also the cPLA$_2$ inhibitors MAFP and AACOCF$_3$ were shown to reduce thermal hyperalgesia induced by carrageenan- or formalin-induced flinching in rats [56] suggesting that not only the IIA-sPLA$_2$, but also the cPLA$_2$, is involved in the molecular mechanisms of nociception. Furthermore, it was shown that AACOCF$_3$ reduced chronic inflammatory responses in mice. Thus, AACOCF$_3$ given intraperitoneally inhibited phorbol ester-induced chronic ear edema [57]. Oral application of AACOCF$_3$ also prevented the development of airway hyperresponsiveness in a mouse asthma model [57] and reduced acute lung injury induced by septic syndrome in mice [58]. AACOCF$_3$ also had beneficial effects in a mouse model of experimental autoimmune encephalomyelitis, which is an inflammatory demyelinating disease of the central nervous system that results in central nervous system lesions [59]. However, due to the fact that AACOCF$_3$ is not exclusively selective for the cPLA$_2$ [60] and, additionally, a cell lytic activity has been reported [61], the various in vivo activities of AACOCF$_3$ must be viewed with caution. Recently, second generation cPLA$_2$ inhibitors have become available such as 2-oxoamides which exerted potent cPLA$_2$ inhibitory effects in vitro and proved efficiency in first in vivo models of inflammation and pain [62]. All these data strongly suggest that cPLA$_2$ is a valid target to treat inflammatory diseases, and therefore, the development of novel more selective cPLA$_2$ inhibitors is of utmost importance.

AACOCF$_3$ has the characteristic of being a slow binding inhibitor of cPLA$_2$ [21]. The trifluoromethyl ketone group turned out to be especially important for inhibition since substitution of this group by either CONH$_2$, CHO, COCH$_3$ or CH(OH)CF$_3$ led to a loss of inhibitory potency [21]. Holmeide and Skattebol speculated that the replacement of the methylene group β to the carbonyl group of the ketone with a sulphur atom would make the carbonyl carbon more electrophilic and consequently the molecule a more potent inhibitor of cPLA$_2$ [12]. This assumption is indeed confirmed by our study that showed a more potent inhibitory effect of the sulphur-containing AVX001 and

AVX002 when compared to the AACOCF$_3$ which originally was reported to inhibit cPLA$_2$ by 78 % at 1.6 mol % of AACOCF$_3$ in the cPLA$_2$ assay system [63]. A further point that needs attention is the difference of potencies of AVX001 and AVX002 in a cell-free in vitro system compared to the potencies in a cellular system. Riendeau et al. already highlighted the issue that in a cellular system higher concentrations of AACOCF$_3$ were needed when compared to the in vitro system [63]. This is due to the fact that AACOCF$_3$ is a slow binding inhibitor of cPLA$_2$ meaning that it takes many minutes to exert its full inhibitory potency [21]. Since arachidonic acid release in cells is a rapid process taking place within minutes, relatively high concentrations are required to inhibit cPLA$_2$. A second point concerns its stability. Riendeau et al. showed the conversion of the ketone of AACOCF$_3$ to its non-inhibitory alcohol in cells which may especially become relevant in long-term stimulation settings and could reduce their inhibitory potential [63]. Furthermore, polyunsaturated fatty acids are known to be easily oxidized which can additionally reduce their inhibitory effect. Similar explanations may also be true for the AVX001 and AVX002 although the metabolism of these compounds in vivo is still unknown.

In summary, this study has forwarded two novel cPLA$_2$ inhibitors, i.e. the ω3-PUFA derivatives AVX001 and AVX002 which are efficient compounds to reduce inflammatory PGE$_2$ synthesis in mesangial cells. These two compounds are certainly very promising candidates to be tested in in vivo disease models such as in models of chronic inflammatory kidney diseases. Also, further studies are needed to characterize the pharmacokinetic properties of these novel cPLA$_2$ inhibitors AVX001 and AVX002.

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Chapter 5

SUMMARIZING DISCUSSION

This thesis is focused on the role of the extracellular action of S1P in inflammation-associated disorders, and on the determination of the S1P receptor subtype involved in the S1P-triggered proliferation and migration of different cell types including non-cancerous cells, breast cancer cells and their metastatic variants. In addition, we investigated the pharmacological modulation of a cytokine-induced inflammatory reaction in renal mesangial cells.

Accumulating evidence has implicated altered levels of biologically active sphingolipids and enzymes of the sphingolipid metabolism in inflammatory and proliferative disorders, like glomerulonephritis, renal fibrosis, diabetic nephropathy and cancer [6,7]. However, there is still no definitive answer which molecular player in the SphK/S1P/S1PR system is the most favorable target for treating such diseases. In ***Chapter 2***, the role of exogenous S1P and its cognate receptors in inflammation-associated breast cancer metastasis was studied. To investigate the contribution of S1P/S1PRs interaction in malignant progression of breast cancer, we used the breast cancer cell line MDA-MB-231 and two metastatic variants isolated from lung and bone metastases in athymic mice (SCP 4175 and SCP 1833, respectively). Our data reveal that in both metastatic sublines the basal level of $S1P_3$ mRNA expression was significantly increased compared to the parental breast cancer cell line. Moreover, overexpression of $S1P_3$ in metastatic cells contribute to a strong increase of $[Ca^{2+}]_i$ upon stimulation with S1P, although in parental MDA-MB-231cells the effect of S1P was marginal, which provides further evidence that upregulated $S1P_3$ in metastatic cells was functionally active.

Considering the important role of chronic inflammation in cancer progression, we investigated in the metastatic sublines whether eicosanoid-derived metabolites and enzymes were altered compared to the parental cell population. Indeed, our data showed that the basal protein and mRNA level of COX-2 and mPGES1, the enzymes essential for PGE_2 synthesis, were upregulated in lung and bone metastatic, but not in parental cells. Enhanced

COX-2 expression was observed in 40% of invasive breast carcinoma [26] and identified as a biomarker of metastasis to lung and bone [27,28]. Many human cancers exhibit elevated levels of PGE_2 due to the upregulation of COX-2. A direct role of PGE_2 in carcinogenesis has been shown in a number of studies. For instance, weekly i.p. administration of PGE_2 significantly increased the incidence of intestinal adenomas in F344 rats [29]. In addition, another study demonstrated that gavage treatment of Apc^{Min} mice with PGE_2 increased epithelial cell proliferation and adenoma growth via the Ras/MAPK pathway [30]. Here, we report that exogenous S1P induces COX-2 gene expression and subsequent production of PGE_2 in lung and bone metastatic cells, but not in parental MDA-MB-231 cells. Moreover, pharmacological inhibition of $S1P_2$ and $S1P_3$ receptors led to a significant decrease of PGE_2 secretion in the metastatic sublines, suggesting that both receptors are involved. On the other hand, the agonism of $S1P_3$ demonstrated a critical role of this receptor subtype in the overproduction of PGE_2 in the metastatic cells. Additionally, the synthesis of PGE_2 in $S1P_3$-overexpressed MDA-MB-231cells was investigated. According to our previous finding, the COX-2 promoter is silenced in parental MDA-MB-231 cell due to epigenetic modifications. Therefore, exogenous S1P did not exhibit any effect in $S1P_3$-overexpressed MDA-MB-231 cells. However, in the presence of the demethylative agent aza-C S1P demonstrated a strong induction of PGE_2 synthesis in $S1P_3$ overexpressing cells compared to non-transfected cells. These data clearly demonstrate that exogenous S1P via $S1P_2$ and $S1P_3$ can induce the COX-2/ PGE_2 signaling pathway in breast cancer cells.

Cell proliferation, survival and migration represent crucial hallmarks of carcinogenesis and cancer progression. The S1P-mediated effect on these processes is determined by the pattern of receptor subtype expression. According to data reported by Li et al. [13], migration and invasion of Wilms tumor cells are mediated by S1P through the $S1P_1/G_{i/o}$ signaling pathway, while $S1P_2$ activity rather inhibits these effects. Yamashita et al. [14] in their study

demonstrated that exogenous S1P stimulated migration of gastric cancer cells with high expression of $S1P_1$ and/or $S1P_3$. In contrast, migration of melanoma cells, which express $S1P_2$, was inhibited by S1P [15]. Similar effects were observed in glioblastoma cells: cell lines expressing high level of $S1P_2$ did not show enhanced migration in response to treatment with S1P [16]. Surprisingly, while $S1P_2$ generally inhibits migration it can increase glioma invasiveness. $S1P_2$ upregulates urokinase-type plasminogen activator (uPAR) and plasminogen activator inhibitor-1 (PAI-1), which are required for the invasion of U373 cells [17]. In prostate adenocarcinoma, S1P via $S1P_2$ activates the Akt pro-survival signaling pathway [18] and $S1P_2$ expression in chronic myeloid leukemia determines the stability of the BCL-ABL1 fusion protein and as a result increases proliferation [19]. Furthermore, $S1P_2$-regulated ERK1/2 phosphorylation mediates chemotherapeutic resistance of colon cancer cells [20]. In this context, it should not be forgotten that $S1P_2$ can induce the COX-2/ PGE_2 pathway, a key mediator of cancer-associated inflammation [21].

In lung adenocarcinoma cells S1P induces proliferation and invasion through $S1P_3$-mediated expression of the epidermal growth factor receptor (EGFR) [22]. Estrogen receptor positive (ER^+) breast cancer cell lines also respond to S1P via $S1P_3$ with subsequent transactivation of the EGFR [23], and $S1P_3$ expression was associated with poor prognosis and shorter patient survival [24]. Furthermore, in estrogen receptor negative (ER^-) breast cancer cells migration can be initiated via $S1P_4$ and downstream activation of the ERK1/2 pathway [25].

In our study we found that both metastatic sublines, which overexpress $S1P_3$, demonstrated a significantly higher migratory and invasive capacity compared to the parental MDA-MB-231 cells. These effects could be further enhanced by treatment with S1P or PGE_2. In addition, we observed that antagonism of $S1P_3$ prevents S1P-stimulated migration and invasion of metastatic cells whereas $S1P_2$ antagonism has no effect. Moreover, treatment of

cells with the selective COX-2 inhibitor celecoxib disrupted S1P-induced cell migration and invasion. Therefore, the induction of PGE_2 by S1P/$S1P_3$ interaction contributes to the migratory and invasive phenotype of breast cancer cells

To investigate the mechanism by which the S1P activated COX-2/PGE_2 pathway triggers migration and invasion of metastatic cells, the mRNA expression pattern of EP receptors in these cells was studied. All four known EP receptor subtypes are expressed in MDA-MB-231 cells and its lung and bone metastatic sublines. However, particularly EP_2 demonstrated high expression and was strongly upregulated in metastatic cells. Using subtype-specific EP receptor antagonists we identified that both the EP_2 and the EP_4 receptors are involved in S1P-triggered migration and invasion of the metastatic MDA-MB-231 breast cancer cells. Thus, for the first time we here report that upregulation of $S1P_3$ in breast cancer is a potential promoter of metastasis, which increases cell migration and invasion by linking S1P to the COX-2/PGE_2 pathway and subsequent EP_2/EP_4 receptor activation.

An effect of S1P on inflammation and cell migration may have impact not only on cancer progression and metastasis, but also for renal diseases where inflammation and migration are typical features for mesangioproliferative glomerulonephritis. To this end, our next study focused on the role of sphingolipids in proliferation and migration of mouse mesangial cells and fibroblasts. The data demonstrated in ***Chapter 3*** reveal that cells isolated from SphK2 gene deficient mice as well as cells which were transiently transfected with siRNA against SphK2 are able to proliferate and migrate significantly faster when compared with the corresponding wild-type cells. This supports our previous finding that SphK2 has a pro-apoptotic and anti-proliferative functions [8]. Furthermore, we showed that the classical MAPK/ERK and PI3K/Akt signaling cascades are involved in the enhanced proliferative and migratory capacity of SphK2ko cells. The role of small G proteins in cell survival and

migration has been previously described [9]. To this end, here we investigated whether some members of the G protein family are also altered upon loss of SphK2. Indeed, using G-LISA® assay, we found that both the basal and S1P-stimulated RhoA activity were significantly increased in SphK2ko mesangial cells, while other small G proteins (Ras, Rac and cdc42) were not affected by depletion of SphK2.

Moreover, SphK2 deficiency contributes to the enhanced expression of SphK1, which suggests that the compensatory mechanism exist between these two kinases. However, quantification of sphingolipids by LC MS/MS analysis indicated an increase of cellular S1P only in SphK2ko MEFs but not in mMCs, which could be due to the higher activity of S1P transporters in mMCs and thereby to the faster secretion of intracellular S1P.

Numerous studies have addressed the possible role of different S1PR subtypes in cell motility and migration. Katsume et al. [10] demonstrated that S1P mediates proliferation of mesangial cells via $S1P_2$ and $S1P_3$ in a G_i-dependent manner by activation of the ERK1/2 pathway. This finding was supported by another study where the authors also reported on the proliferative capacity of exogenous S1P via S1PRs [11]. Moreover, it has been shown that exogenous S1P, by activating its cognate receptors, induces migration of mesangial cells [12]. To address this, we screened the S1PRs mRNA profile in SphK2ko and wild-type cells. Specifically, $S1P_3$ expression was up-regulated in SphK2ko mMCs and MEFs. Furthermore, depletion of $S1P_3$ in mMCs decreased cell proliferation and migration. Therefore, our study demonstrate that SphK2 has a suppressive effect on cell proliferation and migration, the key cellular responses implicated in chronic kidney inflammatory diseases. At the same time, suppression of SphK2 leads to an increase of $S1P_3$ expression, which favors the possibility that the observed enhanced proliferation and migration upon loss of SphK2 could be due to an autocrine loop initiated by S1P binding

to $S1P_3$. All these finding together support the conclusion that $S1P_3$ is involved in S1P stimulated cell migration/invasion in different cell types.

Finally, in ***Chapter 4*** the inhibitory effect of the ω3-PUFA derivatives AVX001 and AVX002 on $cPLA_2$ activity in renal mesangial cells was studied. Since, activation of PLA_2 represents a crucial step in the generation of eicosanoids and, thereby, in inflammatory responses, targeting of this initial reaction represents a promising approach for anti-inflammatory therapy. Although all PLA_2 isoenyzmes are able to mediate inflammation, the majority of evidences attribute this activity to $sPLA_2$ and $cPLA_2$ [1]. Particularly, $sPLA_2$ is speculated to be involved in general inflammatory responses, while $cPLA_2$ is more associated with chronic inflammation [2]. The present study shows that the novel ω3-PUFA derivatives AVX001 and AVX002 directly inhibit $cPLA_2$ activity in vitro and as a functional consequence efficiently reduce cytokine-stimulated PGE_2 production at low μM concentrations. Previously, Pfeilschifter et al. [3] demonstrated that $sPLA_2$ and $cPLA_2$ isoforms are both involved in cytokine-induced PGE_2 formation. Our data suggest that AVX001 and AVX002 reduce cytokine-stimulated $sPLA_2$ protein and mRNA expression, as well as down-regulate the promoter activity of $sPLA_2$. However, the inhibitory activity of AVX001 and AVX002 compounds on $sPLA_2$ was less potent than the one observed on PGE_2 formation, which could be due to the overlapping and more efficient effect on $cPLA_2$. Mechanistically, we could further demonstrate that $cPLA_2$ is involved in the cross-regulation of $sPLA_2$ expression via NFκB activation. Previous studies also suggested that $cPLA_2$ is involved in NFκB activation [4], in addition, NFκB is involved in $sPLA_2$ gene transcription [5]. Here, we report that inhibition of $cPLA_2$ by AVX compounds prevents NFκB activation by blocking IκB degradation. Also, in our study we could exclude the action of AVX001 and AVX002 through PPAR activation. We demonstrate that, although AVX-inhibited PGE_2 production was partially reversed by the PPARγ antagonist G3335, an even more potent effect of PPAR antagonism was

observed in the absence of the AVX compounds. This supports the conclusion that the stimulatory effect of the PPARγ antagonist on the PGE_2 pathway is rather general than AVX-specific. Summarizing these data, direct inhibition of $cPLA_2$ activity by the novel ω3-PUFA derivatives AVX001 and AVX002 leads to the consequent abrogation of PGE_2 formation, suggesting that their use holds promising for treating inflammatory diseases.

A summary of the S1P-induced effect on the inflammatory response in cells and the induction of proliferation and migration is depicted in Fig. 1.

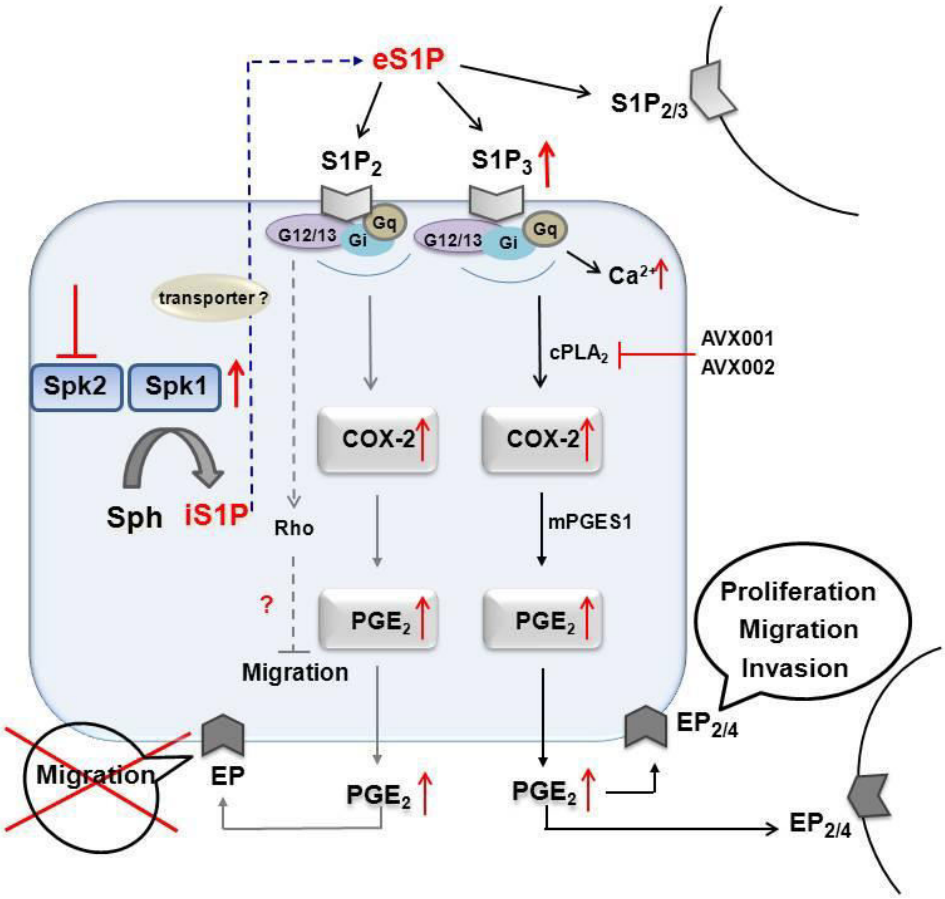

Fig. 1. Schematic overview of signaling pathways initiated by extracellular S1P (eS1P) via its cognate receptors that cause the inflammatory response and subsequent induction of cell proliferation and migration.

Conclusions and perspectives

The work presented in this thesis expand our knowledge about the role of S1P in regulating inflammation and proliferation of cells implicated in the pathogenesis of different inflammatory disorders, including fibrosis and cancer. Another aspect of this work was to identify possible targets for pharmacological intervention to treat these diseases. Here we propose different approaches and several possible molecular targets to design more effective treatments.

A major conclusion of this thesis is that $S1P_3$ receptor upregulation contributes to the enhanced proliferative and migratory capacity of mesangial cells and embryonic fibroblasts, as well as to S1P-induced inflammation and subsequent increase of the metastatic potential of breast cancer cells. These findings confirm the concept that S1P-mediated cellular responses depend on the S1PR expression pattern. Here we found that the $S1P_3$ receptor is not the only family member which involved in inflammatory responses mediated by S1P. Our data obtained in metastatic breast cancer cells clearly demonstrated that $S1P_2$ activation also resulted in increased of PGE_2 formation. Interestingly, the S1P-induced inflammatory response via $S1P_2$ does not alter migration of metastatic cells. The explanation for this phenomenon could be that activation of $S1P_2$ rather inhibits migration by activation of the Rho pathway. Thereby, this data confirms that $S1P_3$, but not $S1P_2$, activation by S1P contributes to breast cancer progression and metastasis. Moreover, the results obtained in studies with non-cancerous cells confirm that upregulation of $S1P_3$ upon loss of SphK2 correlates with a higher proliferative and migratory capacity of cells. Therefore, our findings strongly suggest that the S1P/$S1P_3$ signaling cascade represents a potential target for the therapeutic intervention in inflammatory-associated renal diseases and metastatic breast cancer.

The second major conclusion of this work is that targeting $cPLA_2$ using novel PUFA inhibitors could be an effective alternative approach for preventing cytokine-activated inflammatory response.

Although, our results clearly shed more light on S1P-mediated processes associated with inflammatory and proliferative cellular responses, future studies are warranted to fully understand these processes. Future challenges include characterization of the specific pathophysiological role of the $S1P_3$ receptor, as well as the development of more effective and specific antagonists. For example, preclinical studies using genetically modified mice may help to clarify the contribution of $S1P_3$ to inflammation and proliferation, and to unveil the clinical relevance of the S1P/$S1P_3$ signaling cascade for molecular intervention.

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ACKNOWLEDGMENTS

I would like to give my deepest thanks to my thesis supervisors, Prof. Dr. Uwe Zangemeister-Wittke and Prof. Dr. Andrea Huwiler. I am very thankful for their guidance, for their support and great ideas, which helped me to complete my project. It wouldn't be possible for me to manage to focus on the right topics and succeed in them without constant supervision and inspiring discussions with you. Also, I would like to acknowledge my gratitude to my co-referee, Prof. Dr. med. Oliver Gautschi, and to my mentor, Prof. Dr. Mario Tschan, and all the committee members for their encouragement.

I am sincerely grateful to Prof. Dr. Josef Pfeilschifter, Dr. Doriano Fabbro and Dr. Stefanie Schwalm for their contribution to this work. Special thanks to Dr. Nancy Hynes, Friedrich Miescher Institute of Biomedical Research, for providing the MDA-MB-231 cell lines and its metastatic variants 4175 and 1833.

Specially, I would like to acknowledge the support and help of Dr. Oleksandr Pastukhov, who played a crucial role in my professional and personal formation. I am thankful to the group members with whom I started to work and who helped me a lot to do the first steps in my PhD career: Dr. Nataliya Kotelevets, Dr. Faik Imeri, Daniel Fallegger, Kevin Oberson and Marianne-van Laer. Also, many thanks to my colleagues with whom I came to the finish: Olivier Blanchard, Aurelio Jenni, Bisera Stepanovska and Luca Reali. It was really nice to work with them. Special thanks to my labmate and friend Tankica Maneva Timcheva that she shared with me all the hard and nice times during my PhD life and that I could always rely on her.

Finally, warmest thanks to my family: my mother Nina and my father Vitaliy, and to Dmitriy for their love, patience and mental support in all the situations of my life. I am also thankful to my friends. Without all the people I mentioned above I wouldn't be able to achieve this goal.

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EDUCATION

2011 – present	PhD program in Biomedical Sciences, Institute of Pharmacology, University of Bern. Thesis title – "The role of S1P3 receptor subtype in inflammation-associated proliferative disorders"
2009 – 2010	Master of Science in Pharmacy. Awarded Master degree with Honors, Pharmaceutical Faculty, National University of Pharmacy, Kharkiv, Ukraine. Diploma thesis – "Pharmacological study of a new combined cardioprotector drug „Novocratal".
2005 – 2009	Bachelor in Pharmacy. Pharmaceutical Faculty, National University of Pharmacy, Kharkiv, Ukraine. Diploma thesis – "Consumption and cost-effectiveness analyses of penicillins when used in clinical practice".

ADDITIONAL EDUCATION

2016	GCP – Essential Guidelines and Legislation 2016 (PharmaSchool, online course)
2013 – 2015	German language studies in Alemania Deutschschule
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PROFESSIONAL EXPERIENCE

2011 – 2016	PhD student, Institute of Pharmacology, University of Bern, Switzerland; research in the field of Molecular Biology of Breast cancer metastasis
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2009 – 2010	Internship as a pharmacist in the Central Regional Pharmacy № 195, Kharkiv, Ukraine

ACTIVE PARTICIPATION IN NATIONAL/INTERNATIONAL MEETINGS

- **Filipenko I**, Huwiler A, Zangemeister-Wittke U (poster): The $S1P_3$ is overexpressed in lung and bone metastatic breast cancer cells and increases tissue invasion by stimulation of prostaglandin E_2 synthesis. 14thInternatioal Summer School 26 – 28.07.2015, Bönigen, Switzerland

- Timcheva TM, **Filipenko I**, Schwalm S, Ebadi M, Hofmann LP, Zangemeister-Wittke U, Pfeilschifter J, Huwiler A (poster). Sphingosine kinase 2 deficiency increases proliferation and migration of renal mesangial cells and fibroblasts. Spring Meeting 2015 of the Swiss society of Pharmacology and Toxicology (SSPT), April 23rd, 2012 Bern, Switzerland
- **Filipenko I** (talk): The role of S1P3 receptor subtype in cancer metastasis. GCB Students' Symposium 2015, January 28, 2015 Bern, Switzerland
- **Filipenko I** (talk): The role of $S1P_3$ receptor subtype in metastasis. 13thInternatioal Summer School 27 – 29.07.2014, Stein am Rhein, Switzerland
- **Filipenko I**, Huwiler A, Zangemeister-Wittke U (poster): The role of sphingolipids in cancer inflammation and metastasis. GCB Students' Symposium 2015, January 29, 2014 Bern, Switzerland
- **Filipenko I** (talk): The role of sphingolipids in cancer inflammation and metastasis. 12thInternatioal Summer School 4 – 6.08.2013, Vevey, Switzerland
- **Filipenko I**, Simon M, Flütsch A, Kotelevets N, Bourquin F, Grütter MG, Huwiler A, Zangemeister-Wittke U (poster). Espilase, recombinant form of the S1P lyase, in an attractive novel drug candidate to treat proliferative and fibrotic diseases. Spring Meeting 2012 of the Swiss society of Pharmacology and Toxicology (SSPT), April 19th, 2012 Bern, Switzerland

LANGUAGES

Ukrainian and Russian (native languages)
English (fluent)
German (pre-intermediate)

LIST OF PUBLICATIONS

- Huwiler A, Feuerherm AJ, Sakem B, Pastukhov O, **Filipenko I**, Nguyen T, Johansen B: The ω3-polyunsaturated fatty acid derivatives AVX001 and AVX002 directly inhibit cytosolic phospholipase A(2) and PGE(2) formation in mesangial cells. J B Pharmacol. 2012 Dec, 167 (8): 1691 – 701
- Schwalm S, Timcheva TM, **Filipenko I**, Ebadi M, Hofmann LP, Zangemeister-Wittke U, Pfeilschifter J, Huwiler A: Sphingosine kinase 2 deficiency increases proliferation and migration of renal mouse mesangial cells and fibroblasts. Biol Chem. 2015 Jun, 396 (6-7): 813 – 25
- **Filipenko I,** Schwalm S, Reali L, Pfeilschifter J, Fabbro D, Huwiler A, Zangemeister-Wittke U: Upregulation of the $S1P_3$ receptor in metastatic breast cancer cells increases migration and invasion by induction of PGE_2 and EP_2/EP_4 activation. Biochimica et Biophysica Acta (BBA) – Molecular and Cell Biology of Lipids. 2016 Nov, 1861 (11): 1840 – 51

Manuscripts published in Ukraine:

- Yakovleva L, Matyashova N, **Filipenko I**: Pharmacoepidemiological evaluation of consumption penicillin antibiotics by using ATC / DDD-methodologies. Rational pharmacotherapy. 2010, 4: 1 – 3
- Laryanovskaya Y, **Filipenko I**, Grubnic I: The experimental research of pharmacological activity a gel-cream “Molozol”. Problems of ecological and medical genetics and clinical immunology. 2012, 4 (112): 308 – 314

Declaration of Originality

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I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 29.09.16

Signature