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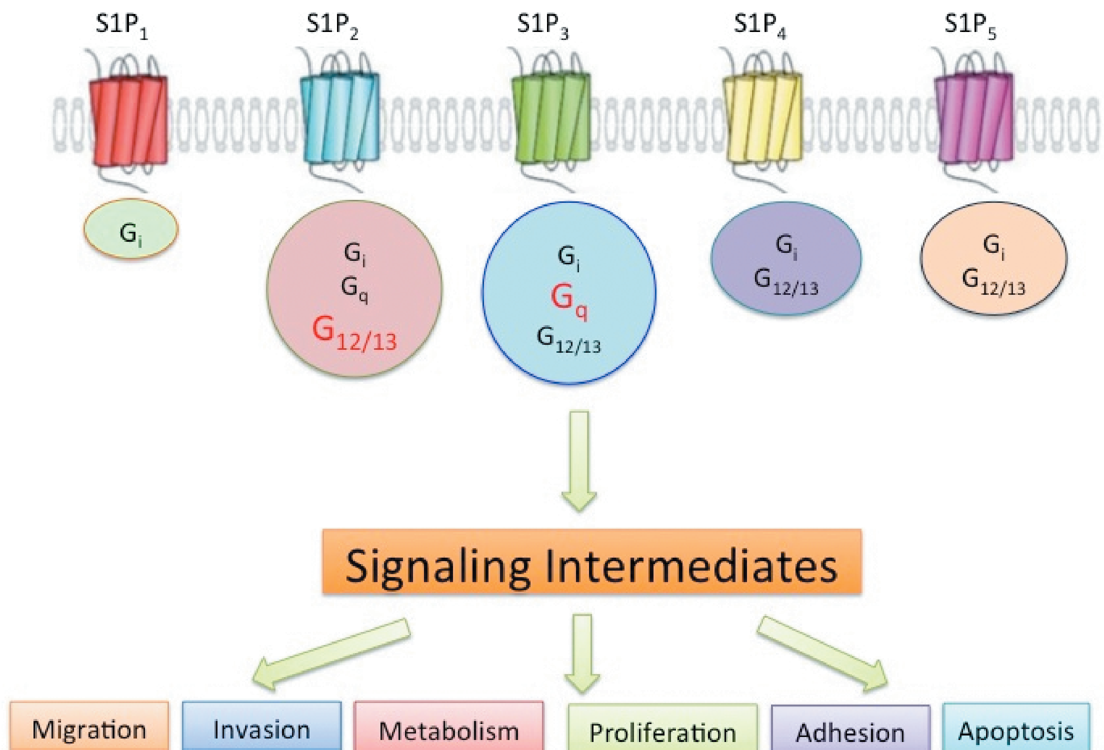
Sphingosine-1-Phosphate-Evoked Invasion of Follicular Thyroid Cancer Cells:

Evidence for the Involvement of HIF-1α, MMP2 and MMP9



SPHINGOSINE-1-PHOSPHATE-EVOKED INVASION OF FOLLICULAR THYROID CANCER CELLS: EVIDENCE FOR THE INVOLVEMENT OF HIF-1α, MMP2 AND MMP9

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ABSTRACT

Sphingolipids are widely expressed molecules, which traditionally were considered to have majorly structural properties. Nowadays, however, they are implicated in a wide range of different biological processes. The bioactive lipid sphingosine 1-phosphate (S1P) has emerged during the past decade as one of the most studied molecules due to its proliferative and pro-migratory abilities both during normal physiology and in the pathology of a subset of different diseases. Migration and invasion of cancer cells require changes in cell behavior and modulation of the tissue microenvironment. Tumor aggressiveness is markedly enhanced by hypoxia, in which hypoxia inducible transcription factors 1-2α (HIF-1-2α) are activated to promote metabolism, proliferation and migration. Invasion requires degradation of the extracellular matrix (ECM) achieved by several degrading and remodeling enzymes. Matrix metalloproteinases (MMPs) are broadly expressed and well accepted as proteolytic enzymes with essential roles both in normal physiology and in pathology.

Previously, S1P was shown to strongly evoke migration of follicular ML-1 thyroid cancer cells. The objective of this study was to further investigate and understand the mechanisms behind this regulation. In the first project it was demonstrated that S1P enhances the expression and activity of HIF-1α. S1P enhanced the expression of HIF-1α by increasing its synthesis and stability. The S1P-increased HIF-1α was mediated via $S1P_3$, $G_{i/0}$, PI3K, PKCβI, ERK1/2, mTOR and translation factors p70S6K and eIF4E. Finally, it was shown that HIF-1α mediated S1P-induced migration.

The ECM is constituted of a complex and coordinated assembly of many types of proteins. In order to be able to invade, cells need to break down the ECM, therefore several key players in this event were investigated in the second project. S1P increased the secretion and activity of MMP2 and MMP9 via S1P-receptor 1 and 3 and that these MMPs participated in the S1P-facilitated invasion of ML-1 cells. In this interplay, calpains and Rac1 were involved, both of which are crucial players in migration and invasion.

The prognosis for some types of thyroid cancer is relatively good. However, there are forms of thyroid cancers, for which there are no treatments or the current available treatments are inefficient. Thus, new medical interventions are urgently needed. In the third project the significance of the S1P-receptor modulating drug FTY720, which is currently used for the treatment of multiple sclerosis (MS), was studied. The effect of FTY720 was tested on several thyroid cancer cell lines, and it inhibited the proliferation and invasion of all cancer cell lines tested. In ML-1 cells, FTY720 attenuated invasion by blocking signaling intermediates important for migration and invasion of the cells. Moreover, FTY720 inhibited the proliferation of ML-1 cells by increasing the expression of p21 and p27, hence, inducing cell arrest in G1 phase of the cell cycle. Thus, it can be suggested that FTY720 could be used in the treatment of thyroid cancer.

LIST OF ORIGINAL PUBLICATIONS AND MANUSCRIPT

This thesis is based on the work from two original publications and one submitted manuscript, which are referred to in the text by their Roman numbers.

I **Kalhori, V***., Kemppainen, K*., Asghar, M.Y., Bergelin, N., Jaakkola, P., Tornquist, K., 2013. Sphingosine-1-Phosphate as a Regulator of Hypoxia-Induced Factor-1alpha in Thyroid Follicular Carcinoma Cells. PLoS One 8, e66189.

II **Kalhori, V**., Tornquist, K., 2015. MMP2 and MMP9 participate in S1P-induced invasion of follicular ML-1 thyroid cancer cells. Mol. Cell. Endocrinol. 404, 113-122.

III **Kalhori, V**., Magnusson, M., Asghar, M.Y., Pulli, I., Törnquist, K., 2015. FTY720 (Fingolimod) attenuates basal and sphingosine 1-phosphate-evoked thyroid cancer cell invasion. Manuscript is submitted.

* Equal contribution.

ABBREVIATIONS

Akt	Protein Kinase B
AMF	Autocrie Motility Factor
ARD	Arrest-defective
ARIH	Autosomal Recessive Icthyosis with Hypotrichosis
ATC	Anaplastic Thyroid Carcinomas
BMP1	Bone Morphogenetic Protein 1
cIAP2	Cellular Inhibitor of Apoptosis
DTC	Differentiated Thyroid Carcinomas
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ERK1/2	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factors
FIH	Factor Inhibiting HIF
FTC	Follicular Thyroid Carcinoma
GPCR	G-protein Coupled Receptor
GPI	Glycosylphosphatidylinositol
HAI-1	Hepatocyte growth factor Activator Inhibitor-1
HDAC	Histone Deacetylase
HDL	High-Density Lipoprotein
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Response Element
Hsc70	Heat shock cognate protein 70
HUVEC	Human Umbilical Vein Endothelial Cells
LOX	Lysyl Oxidase
MAPK	Mitogen-Activated Protein Kinase
MDM2	Mouse Double Minute 2 Homolog
MEF	Mouse Embryonic Fibroblasts
MEN	Multiple Endocrine Neoplasia
MMP2	Matrix Metalloproteinase 2
MMP9	Matrix Metalloproteinase 9
MS	Multiple Sclerosis
MT1-MMP	Membrane Type-1 Matrix Metalloproteinase
MTC	Medullary Thyroid Carcinoma
mTOR	Mammalian Target of Rapamycin

ODDD	Oxygen-Dependent Degradation Domain
p-AKT	Phospho-Protein Kinase B
p-ERK1/2	Phospho-Extracellular Regulated Kinase
p-mTOR	Phospho-Mammalian Target of Rapamycin
p-p70S6K	Phospho-Protein 70S6 Kinase
p-VEGFR2	Phospho Vascular Endothelial Growth Factor Receptor 2
p70S6K	Protein 70S6 Kinase
PAR2	Protease Activated Receptor 2
PAX-8	Paired box gene 8
PDGF	Platelet-Derived Growth Factor
PHD	Prolyl Hydroxylase
PKA	Protein Kinase A
PKCα	Protein Kinase C Alpha
PKCβ	Protein Kinase C Beta
PLC	Phospholipase C
PPARϒ1	Peroxisome Proliferator-Activated Receptor ϒ
PTC	Papillary Thyroid Carcinoma
PTEN	Phosphatase and Tensin Homolog
PTH	Parathyroid Hormone
RACK1	Receptor for Activated C kinase 1
RET	Rearranged during Transfection
RIP	Receptor Interaction Protein
ROCK	Rho-associated Protein Kinase
S1P	Sphingosine-1-Phosphate
$S1P_1$	Sphingosine-1-Phosphate receptor 1
$S1P_2$	Sphingosine-1-Phosphate receptor 2
$S1P_3$	Sphingosine-1-Phosphate receptor 3
$S1P_4$	Sphingosine-1-Phosphate receptor 4
$S1P_5$	Sphingosine-1-Phosphate receptor 5
SK	Sphingosine Kinase
T3	Triiodothyronine
T4	Thyroxin
TGF	Tissue Growth Factor
TIMP	Tissue Inhibitors of Matrix Metalloproteinase
TSH	Thyroid-Stimulating Hormone
uPA	Urokinase Plasminogen Activator

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1. INTRODUCTION

Sphingosine-1-phosphate (S1P) is a bioactive lipid with the ability to induce migration, invasion, proliferation and angiogenesis. Thus, S1P plays a fundamental role in tumor biology. S1P elicits its effects by binding to one of its five highly specific G-protein coupled receptors termed $S1P_{1-5}$ and activating different G-proteins. Worth keeping in mind is that the ultimate outcome of the S1P stimulation, however, depends completely on the S1P receptor and the type of G-protein it activates. Thus, S1P can give rise to both stimulatory and inhibitory responses. In endocrine organs, thyroid cancer is one of the most commonly occurring types of cancer. Our group has for almost a decade studied the effect of S1P on several cellular processes in several thyroid cancer cell lines. We showed earlier that S1P very strongly enhances the migration of the follicular ML-1 thyroid cancer cells via $S1P_{1,3}$ and $G_{i/0}$ proteins. In this regulation the vascular endothelial growth factor receptor 2 (VEGFR2) plays an essential role. Thus, a cross-talk between these two receptor systems exists in ML-1 cells.

Tumor environment is usually hypoxic, thus tumor cells suffer from oxygen deficiency, a condition extremely unfavorable to the cancer cells. Hence, lack of oxygen could in fact be very fatal to the cells. However, cancer cells have tackled this problem by inducing a family of hypoxia inducible transcription factors (HIF-1-2α), which enhance metabolism and survival of the cancer cells. HIF-1α has gained a substantial amount of attention due to its ability to activate a subset of different genes promoting cancer cell survival, proliferation, migration, invasion and angiogenesis.

One hallmark of tumor biology is invasion, which is a multistep process requiring changes in cell behavior and in the environment around the tumor and the invading cell. The first requirement for a cell to be able to invade is loss of cell-cell and cell-extracellular matrix (ECM) contacts. Hence, invasion requires degradation of the ECM, in which matrix metalloproteinase (MMPs) act as one of the main governors.

The pro-angiogenic molecule S1P has been shown in different studies to enhance tumor growth and metastasis of several types of cancers by modulating key players in these cellular processes. Thus, inhibition of S1P receptors has become an attractive target in the treatment of different diseases. Currently, several different S1P-receptor modulating drugs are in clinical trials. However, only FTY720 has successfully reached clinical practice for the treatment of multiple sclerosis (MS). Nonetheless, FTY720 has been tested in cancer cells, and it is found that it inhibits both migration and proliferation of cancer cells, representing new therapeutic strategies and clinical advantages.

2. REVIEW OF THE LITERATURE

2.1 Thyroid structure and function

As one of the largest endocrine organs, the thyroid gland is situated on the anterior side of the neck. It is composed of two lobes (left and right) connected with each other via an isthmus (a wall between the two lobes). Within the posterior region of the gland four small parathyroid glands responsible for the production of parathyroid hormones (PTH) can be found. Two types of cells can be found in the thyroid tissue: follicular cells, also called thyrocytes, producing thyroxin (T4) and triiodothyronine (T3) hormones, and parafollicular cells, which produce calcitonin. Due to its ability to produce hormones influencing many central functions in the body, the thyroid gland is considered one of the master regulators of biological processes, particularly during development and childhood (Stathatos, 2012). Figure 1 shows the structure and location of the thyroid gland.

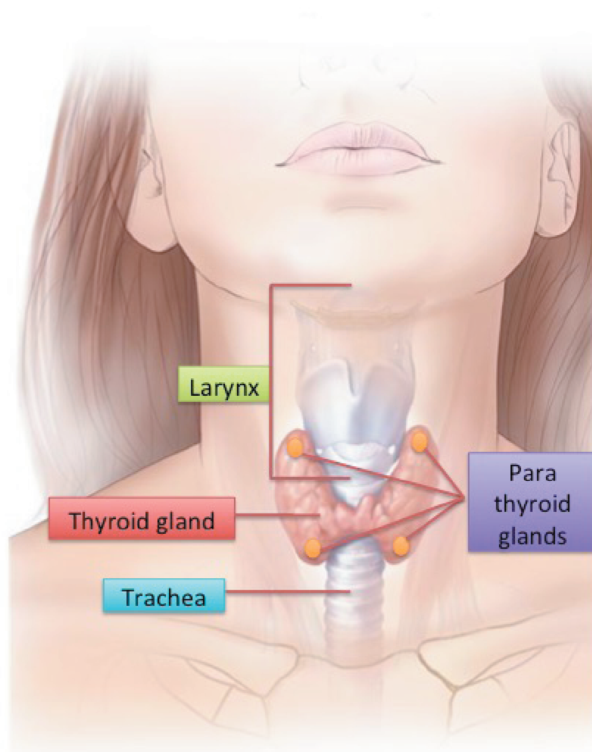

Figure 1. The thyroid gland is situated in the anterior region of the neck, containing four posterior small parathyroid glands. (Adapted and modified from http://www.cancer.gov/types/thyroid/patient/thyroid-treatment-pdq#section/all).

The main function of calcitonin is to regulate the metabolism of calcium. Calcitonin acts to decrease the Ca^{2+} ion concentrations in the blood by different means; it modulates the osteoblastic and osteoclastic activity in the bone, inhibits intestinal absorption of Ca^{2+} and stimulates the excretion of Ca^{2+} by the kidney due to its ability to inhibit the renal tubular cell reabsorption of Ca^{2+}. In contrast to calcitonin, PTH acts to increase the concentrations of Ca^{2+} in the blood. However, PTH itself is regulated via a negative feedback loop, meaning that high concentrations of blood Ca^{2+} prevent the secretion of PTH (Economidou et al., 2011).

The thyroid hormones T3 and T4 are produced from iodine and thyroglobulin by the follicular cells. These hormones are involved in regulation of body metabolism, protein synthesis, thermoregulation and development, in particular the development of the brain. The thyroid-stimulating hormone (TSH) generated and released by the anterior pituitary regulates the synthesis and release of T3 and T4. T4 is synthesized upon iodination of thyroglobulin by the action of thyroperoxidase. However, T4 is the inactive hormone, which in response to deiodinase is converted to the active T3 in the blood (Economidou et al., 2011).

2.1.1 Thyroid diseases

As any other organ of the body, also the thyroid gland is subject to several different types of abnormalities and malfunctions, ranging from a harmless enlargement of the gland (goiter) to highly malignant and deadly cancers. The disorders that strike the thyroid gland are hyperthyroidism, hypothyroidism, thyroiditis, thyroid nodules and cancers (Hong et al., 2015).

Hyperthyroidism is characterized by excessive amounts of thyroid hormones. In general, hyperthyroidism originates from Graves'disease, which is an autoimmune disorder. In this condition, the body produces high amounts of thyroid-stimulating antibodies resulting in excessive production of thyroid hormones. In contrast to hyperthyroidism, hypothyroidism occurs commonly due to insufficient production of the thyroid hormones because of a dysfunctional thyroid gland. However, this disorder can also appear due to iodine deficiency or the autoimmune disease Hashimotos thyroiditis. Furthermore, there are many other factors causing hypothyroidism such as injury to the hypothalamus or the anterior pituitary and previous surgical removal of the thyroid gland. Other types of abnormalities found in the thyroid gland are thyroiditis and goiter. Thyroiditis is an infection of the thyroid tissue caused by bacteria, fungi and viruses mostly affecting children, but also adults can suffer from this disorder. Goiter, which is detected in the front of the neck, is a condition where the thyroid gland is enlarged due to iodine deficiencies and malfunctions of the thyroid gland. Goiter is a harmless condition when treated properly, however, it can be toxic to the patient (Hong et al., 2015).

2.1.2 Thyroid nodules and neoplasms

Thyroid cancer is the most common type of malignancy affecting the endocrine organs, and the thyroid cancer incidences have increased significantly worldwide during the

past decades. Females are known to be more prone to thyroid cancer diseases than men, perhaps due to the female hormones. The reasons for thyroid cancer occurrence are poorly understood. However, many predisposing risk factors have been associated with the disease and are considered to contribute to the development of thyroid cancers. The risk factors include family history, iodine deficiency, exposure to radiation gender and age. In addition to these risk factors, inflammation conditions in the thyroid gland can, if not properly treated in time, lead to development of cancers (Kondo et al., 2006; Jin et al., 2013; Hong et al., 2015).

Usually the thyroid tissue contains small benign nodules, however, they are found quite rarely in the thyroid gland of persons under the age of 20. Thus, when found at the age of 20, the nodules have a higher likelihood of becoming malignant. Often the thyroid nodules present in the thyroid gland are detected by coincidence, either by the patient itself or the physician. However, with advanced modern technology and the knowledge in the field of tumor biology, we have better diagnostic tools for the detection and the identification of thyroid cancer. The most common ways to diagnose thyroid neoplasms are blood tests, ultrasound imaging and fine needle biopsies (FNB). The first symptoms of thyroid cancer are enlarged lymph nodes, which is followed by pain in the anterior side of the neck and hoarseness (Kondo et al., 2006; Jin et al., 2013; Hong et al., 2015).

There are many types of thyroid cancer, ranging from very easy-treating forms to life threatening and deadly ones. The classification of the thyroid carcinomas is based on the origin, stage and degree of metastasis. The thyroid cancer diseases are divided into three main groups consisting of medullary thyroid carcinomas (MTC), anaplastic thyroid carcinomas (ATC) and differentiated thyroid carcinomas (DTC). DTC consists of two subtypes: follicular thyroid carcinoma (FTC) and papillary thyroid carcinoma (PTC). The distinct characteristics for the particular types of thyroid cancers are the degree of differentiation and the mutations occurring in genes specific for them (Xing, 2013).

2.1.2.1 Papillary thyroid carcinoma (PTC)

PTC is the most common type of thyroid cancer with increased incidences attributing to the overall increase in thyroid cancer diseases. PTC is highly differentiated and originates from the follicular thyroid cells accounting for approximately 85% of all thyroid cancer cases. Patients with PTC have a favorable and good overall prognosis (Jin et al., 2013). Biomarkers and molecular and genetic alternations for PTC have been identified. The most frequent gene alternation in PTC is the BRAF V600E mutation occurring in almost 45% of the patients (Nikiforov et al., 2011). Frequent rearrangements in the rearranged during transfection (RET) gene is found in patients with PTC due to the interactions between the intracellular tyrosine kinase domain of RET and the N-terminal domain of various target genes (Nikiforov, 2008). Both RET rearrangements and BRAF V600E mutations lead to a constitutive stimulation of the mitogen-activated protein kinase and extracellular regulated kinase 1/2 (MAPK/ERK1/2) pathway, enhancing cellular growth and metastasis (Nikiforov et al., 2008 and Nikiforov, 2011).

2.1.2.2 Follicular thyroid carcinoma (FTC)

Being the second most frequently occurring type of thyroid cancer, FTC accounts for almost 11% of all thyroid malignancies in countries with iodine sufficiency, and approximately 25-40% in iodine-insufficient areas. The overall prognosis for patients with FTC is good. FTC has its origin in the follicular thyroid cells and is highly differentiated (Jin et al., 2013). FTC has been studied at molecular level, and it has been demonstrated that gene alternations play a crucial role in the pathogenesis of FTC. Both mutations and chromosomal translocations can occur in FTC causing uncontrolled cell growth and proliferation. The most common mutations occur in the RAS-family genes (N-RAS, K-RAS and H-RAS). These mutations are detected in approximately 40% of the patients. Mutations in RAS are normally associated with uncontrolled growth and proliferation (Downward, 2003). In addition, mutations in p53, c-myc, fos, TSH and the PTEN suppressor gene increase the likelihood for occurrence of FTC (Fuhrer et al., 2003). Paired box gene 8 (PAX-8) encodes the peroxisome proliferator-activated receptor ϒ (PPARϒ1), and it is important for thyroid cell growth. In approximately 50% of all cases rearrangements in PAX8/PPARϒ1 has been detected and associated with uncontrolled cell growth (Martelli et al., 2002). Apart from mutations and gene translocations, numerous studies have demonstrated enhanced expression of MicroRNAs in FTC, altering gene transcription and influencing cell proliferation and apoptosis (Weber et al., 2006).

2.1.2.3 Anaplastic thyroid carcinoma (ATC)

ATC is considered as one of the most aggressive types of cancers affecting the human body. It is rare and represents only about 2% of all thyroid cancer cases. ATC is extremely pathogenic associated with poor prognosis and high degree of mortality (Smallridge & Copland, 2010). The survival rate for patients with ATC is extremely low. In most cases patients with ATC have 1-year survival. However, in rare cases patients might survive for 5 years (Broome et al., 2009). ATC derives from de-differentiations of FTCs and PTCs. However, it does not harbor any functional characteristics of its descendent, the follicular cell. Furthermore, ATCs can develop from head and neck squamous cell carcinomas due to the differentiation ability of transcription factor PAX8 (Bishop et al., 2011). As with the other types of thyroid carcinomas, ATCs harbor genetic alternations such as mutations in the genes involved in growth, metastasis and apoptosis. Usually, mutations are found in the genes for p53, RAS, BRAF, the E3 ubiquitin-protein ligase (MIB-1), axin 1, β-catenin and phosphoinositide 3-kinase (PI3K) (Smallridge, 2012). In addition, overexpression of cyclins D1 and E1 has been observed in ATC, boosting cell growth (Kondo et al., 2006).

2.1.2.4 Medullary thyroid carcinoma (MTC)

MTC originates from the parafollicular C cells responsible for the production of calcitonin, thus, highlighting the suitability of calcitonin levels as a marker for MTC. MTCs occur very rarely and account for approximately 5% of all thyroid cancer

incidences. It has a good prognosis when detected in time and the overall survival rate is 5-10 years. However, a poor prognosis is associated with advanced age of the patient, the stage of the tumor, multiple endocrine neoplasia 2B (MEN 2B) and previous neck surgery (van Veelen et al., 2009; Krampitz & Norton, 2014). MTCs can occur sporadically, however, 25% of all reported MTC cases are family-related. Familial MTC emerges as a consequence of familial multiple endocrine neoplasia, MEN 2A and MEN 2B and also familial non-MEN syndrome. Furthermore, alternations in the RET gene have been detected in patients with MTC, and family members of MTC patients are encouraged to undergo examinations and DNA-testing for mutations in the RET gene to secure diagnosis at an early stage as possible (Krampitz & Norton, 2014).

2.2 Tumor transformation, migration and invasion

Giving the complexity of biological processes, cell migration is one of the most interesting phenomena of a living cell, involving several distinct steps, which are orchestrated by a subset of different regulatory mechanisms. Cell migration requires reshaping of the cell accomplished by cytoskeletal rearrangements as well as formation of membrane structures, such as lamellipodia and filopodia. The first step of migration is formation of a front (leading) edge and a rare (back) of the cell. Within the leading edge actin polymerization is thought to be the main force driving the cell forward. The small GTPases CDC42 and Rac1 are usually found in the leading edge, whereas RhoA is found in the rare side of the cell (trailing edge). Within the leading edge focal adhesions assemble, while disassembly takes place in the trailing edge. These processes involve the action of adhesion proteins and proteolytic enzymes (Labelle & Hynes, 2012; Vanharanta & Massague, 2013; McAllister & Weinberg, 2014; Sevenich & Joyce, 2014).

Tumorigenesis is a process where normal cells acquire features characteristic for cancer. Epithelial to mesenchymal transition (EMT) is one hallmark of this transformation, in which cells acquire properties that offer extremely favorable conditions to the transformed cells for survival. After years of investigations, it is now becoming more evident that cells that undergo EMT exhibit features similar to stem cells (Labelle & Hynes, 2012; Vanharanta & Massague, 2013; McAllister & Weinberg, 2014; Sevenich & Joyce, 2014).

Metastasis is a multistage process involving alternations of the tumor microenvironment. The process comprises several critical events such as loss of cell-cell and cell-extracellular matrix (ECM) contacts, degradation of the basement membrane and the ECM, as well as adhesion at the site of the newly invaded tissue. Integrins and cadherins are adhesion proteins influencing the intimate contacts between cells and cell-ECM. In order to be able to migrate from the tissue of origin, cells need to brake down the extracellular matrix. This process requires the involvement of diverse biochemical events and signaling molecules. The journey of the invasive tumor cell is very complex. After loss of cell contacts and the proteolysis of the ECM, cells first intravasate into blood vessels and are transported away from the tissue of origin via the blood. Later on

cells escape from the blood vessels, a process called extravasation. Nonetheless, tumor dissemination and metastasis can also occur through lymphogenesis. Most importantly, the final step of invasion is colonization, meaning adherence of the cells to the newly invaded tissue. At this stage the invasive tumor cells undergo a critical period due to their need for nutrition and survival. Thus, formation of new blood vessels, a process termed angiogenesis, is very vital (Labelle & Hynes, 2012; Vanharanta & Massague, 2014; McAllister & Weinberg, 2013; Sevenich & Joyce, 2014).

2.3 Sphingolipid metabolism

Sphingolipids, also called glycosylceramides, were found already in 1870s. They are a group of lipids containing a conserved aliphatic amino alcohol sphingoid backbone. They were first identified as structural components of lipid membranes. However, later on, a great deal of attention was attributed to the sphingolipids due to their fundamental and diverse functional roles in biological processes in the cell. They are also important phospholipid species in the structure of all plasma lipoproteins.

The bioavailability of sphingolipids is dependent on the unique inter-conversion of the existing metabolites within the sphingolipid group. The biosynthesis of sphingolipids is a result of several highly distinct and complex steps of metabolic reactions tightly regulated by degrading and generating enzymes (Alshaker et al., 2013; Zu Heringdorf et al., 2013). The starting point of the sphingolipid catabolism is sphingomyelin, the major representative among sphingolipids. Sphingomyelin is converted to ceramide by the action of sphingomyelinase. Ceramide is considered a central metabolite in the metabolism of sphingolipid, and can also be formed from condensation of palmitate and serine. Sphingomyelinsyntases are responsible for the conversion of ceramide to sphingomyelin. The pro-apoptotic sphingosine is generated from ceramide, a reaction catalyzed by ceramidases. Sphingosine-1-phosphate (S1P) is generated as a result of phosphorylation of sphingosine by the action of sphingosine kinases 1 and 2 (SK1 and SK2). Depending on the circumstances, S1P can either be converted back to sphingosine by S1P phosphatases, or irreversibly degraded by S1P lyases to palmitaldehyde (hexadecenal) and ethanolamine phosphate species (Pyne & Pyne, 2013; Kerage et al., 2014; Mendelson et al., 2014).

Several years of research and lines of evidence have demonstrated an extremely important role of the sphingolipids in cell function. Particularly, sphingosine, ceramide and S1P are considered master regulators of cell fate. Hence, a rheostat has been suggested with sphingosine and ceramide driving cells into death, and S1P inducing cell proliferation and survival. As can be expected, any disruption in the sphingolipid metabolic pathway can have serious consequences. Therefore, each and one of the components involved in the pathway are regulated very tightly (Pyne & Pyne, 2013; Zu Heringdorf et al., 2013; Kerage et al., 2014;). Figure 2 summarizes the major players in the sphingolipid metabolism.

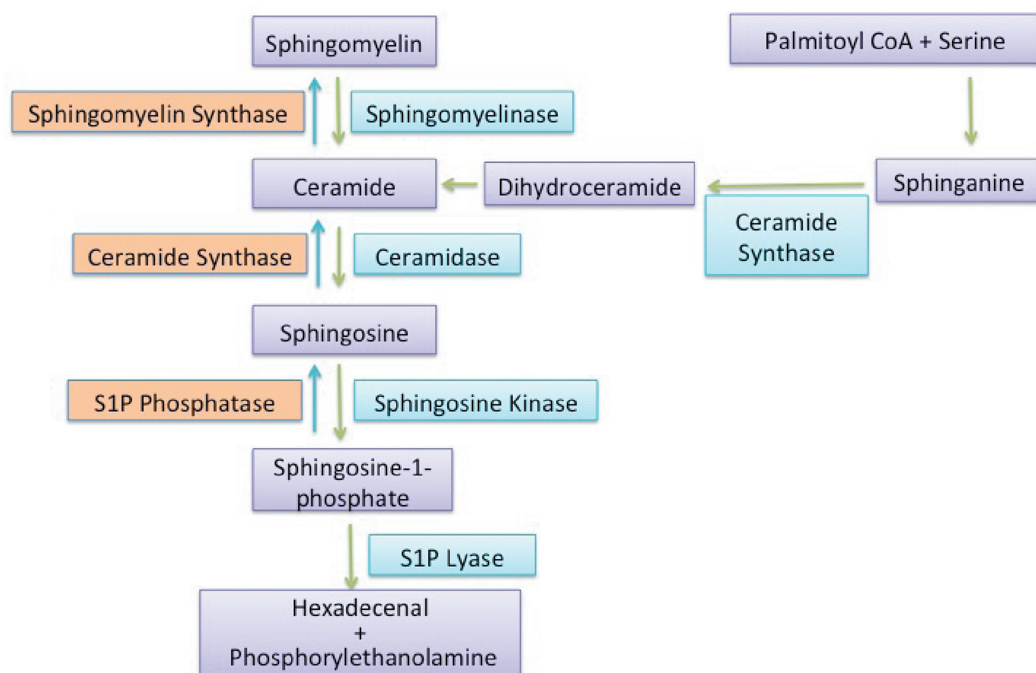

Figure 2. Sphingolipid metabolism. The figure shows different steps and conversions of bioactive sphingolipids. (Adapted from Pyne & Pyne, 2013).

2.3.1 Sphingosine-1- phosphate and its receptors

2.3.1.1 Sphingosine-1-phosphate

Virtually all types of cells in the body can produce sphingosine-1-phosphate (S1P), however, the main sources of S1P are platelets, erythrocytes and immune cells. Upon production, S1P is transported out from the cell by the aid of several transporters in the plasma membrane (Figure 3 demonstrates the structure of S1P). The ATP-binding cassette transporters (ABC) and spinster 2 (spins 2) are the most known and studied transporters of S1P. S1P is presented in high concentrations in the blood and plasma, usually bound to lipoproteins and albumin (Sattler & Levkau, 2009; Rodriguez et al., 2009). The intracellular S1P concentration is very low, but the concentration of S1P in the extracellular milieu is high. These differences in the concentration of S1P generate a S1P gradient, which is important for the action of S1P (Strub et al., 2010; Takabe & Spiegel, 2014).

S1P has been associated with a wide range of different cellular processes both during development and progression of many diseases. During development S1P contributes to the formation of many organs. The most obvious role of S1P is in the development of the vasculature, the heart and the nervous system. Vascularization is important for the survival of cells and is one hallmark of angiogenesis, in which S1P plays a pivotal role by inducing VEGF secretion and VEGFR2 activation. S1P plays an important role even in inflammation and immunological conditions. Furthermore, S1P is frequently overproduced as a consequence of hyperactivity of SK in several cancer

cells. Thus, S1P influences the behavior of cancer cells by inducing their proliferation and migration (Strub et al., 2010; Takabe & Spiegel, 2014).

Recently intracellular targets of S1P have been identified, demonstrating S1P as a secondary messenger in the cells (Nagahashi et al., 2014). The S1P produced by nuclear SK2 can regulate transcription by binding to and, consequently, inhibiting the histone deacetylases (HDAC1 and HDAC2) (Hait et al., 2009).

Furthermore, the S1P produced by SK1 has been shown to have two intracellular targets involved in cellular growth and apoptosis. S1P has been demonstrated to bind to tumor necrosis factor receptor factor 2 (TRAF2), and to act as a crucial cofactor essential for the activity of E3 ubiquitin ligase (Alvarez et al., 2010). Moreover, cellular inhibitor of apoptosis 2 (cIAP2) is a target of intracellular S1P. The mechanism of action is that S1P binds to and enhances the activity of cIAP2, subsequently leading to inhibition of apoptosis (Harikumar et al., 2014).

2.3.1.2 S1P associated with lipoproteins in serum

S1P is present in serum bound to lipoproteins. Around 60% of S1P is bound to HDL and 10% to LDL and VLDL. The remaining 30% of S1P is carried in albumin. HDL present in plasma contains several different apolipoproteins, of which apolipoprotein M is a crucial component in the binding of S1P to HDL (Rodriguez et al., 2009; Karunar et al., 2011; Christoffersen et al., 2011; Bas et al., 2013). Importantly, high levels of plasma apolipoprotein M have been linked to elevated levels of S1P, and this correlation has been suggested to play a role in the anti-atherosclerotic properties of both S1P and apolipoprotein M (Bas et al., 2013).

Extensive studies have demonstrated that the beneficial effects of HDL, at least in endothelial and vascular smooth muscle cells of the vasculature, are dependent on the S1P that is bound to HDL. HDL-bound S1P has been shown to confer vasculoprotection by inducing the release of nitric oxide and prostacyclin. The release of nitric oxide involves Akt-dependent phosphorylation of endothelial nitric oxide synthase, while the prostacyclin release engages activation of cAMP response element binding protein (CREB) mediated by ERK1/2 and p38 mitogen activated protein kinase. The induction of nitric oxide and prostacyclin by HDL-S1P are of importance in the regulation of many cellular events, such as proliferation, apoptosis, cell adhesiveness, angiogenesis and vascular integrity (Rodriguez et al., 2009).

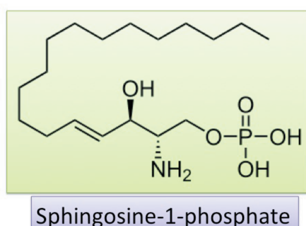

Figure 3. The structure of sphingosine-1-phosphate. (Adapted and modified from http://commons.wikimedia.org/wiki/File:Sphingosine_1-phosphate.svg).

2.3.2 S1P-receptors and mechanism of action

S1P-receptors are a group of G-protein coupled receptors (GPCR) with seven transmembrane helices. Upon activation by their extracellular ligand, GPCRs activate a subset of different G proteins, ultimately leading to stimulation of downstream signaling pathways and a diverse array of cellular responses. After several years of extensive studies, five highly specific S1P-receptors termed $S1P_{1\text{-}5}$, have been identified (Pyne & Pyne, 2013). Almost all types of cells express all or some of the S1P-receptors. Extracellular S1P, such as that associated with HDL-apolipoprotein M, binds S1P-receptors, resulting in activation of different G proteins, and downstream signaling intermediates. However, the outcome of this cascade activation is exclusively dependent on which S1P-receptor and G protein is activated. Hence, S1P-receptors have both opposing and overlapping functions, which is coupled to their distinct G proteins. A substantial amount of evidence has proven S1P/S1P-receptors to be of fundamental importance in physiological processes during development, and also during pathophysiology and progression of many different diseases. Thus, understanding the nature and mode of action of these receptors is of great importance (Dorsam & Gutkind, 2007). The mode of action for the different S1P-receptors is demonstrated in Figure 4.

2.3.2.1 $S1P_1$

$S1P_1$ is the first receptor cloned, and it is expressed in almost all types of tissues, especially in cerebellum and immune cells. Its function, however, has been studied thoroughly in immune cells (Garris et al, 2014). It is a stimulatory receptor highly associated with induction of migration, proliferation, calcium signaling, angiogenesis, invasion and cell survival of both cancerous and non-cancerous cells (Tabasinezhad et al., 2013). Binding of S1P to $S1P_1$ leads exclusively to activation of $G_{i/0}$ resulting in activation of PI3K and Akt pathway, the master regulators of survival of several cell types (Garris et al, 2014). S1P via the same receptor and pathway is able to inhibit H_2O_2-induced apoptosis of granulosa cells (Nakahara et al., 2012). Moreover, $S1P_1$ influences cell proliferation by activating RAS and ERK1/2, while it induces migration and invasion via activation of PI3K and Rac1 (Tabasinezhad et al., 2013). Furthermore, in multiple sclerosis (MS) lesions the expression of $S1P_1$ is up-regulated in astrocytes indicating that astrocytes might function as a new target of anti-inflammatory drugs (Van Doorn et al., 2010). Several lines of evidence have shed light on the collaboration between $S1P_1$ and growth factors. In endothelial cells, S1P is able to activate growth factor receptors as well as to enhance the secretion of growth factors such as VEGF, EGF, PDGF, and bFGF (Tabasinezhad et al., 2013). It is well documented that $S1P_1$ activates integrins to modulate adhesion. $S1P_1$ plays a fundamental role also in modulating the extracellular matrix by affecting several crucial players important for this event. Thus, from a cancer perspective, $S1P_1$ is considered a driving force of tumorigenesis (Tabasinezhad et al., 2013; Pyne & Pyne, 2013).

2.3.2.2 $S1P_2$

Almost all types of cells express $S1P_2$, and in contrast to other S1P receptors it has been demonstrated to exert inhibitory effects on a multitude of biological processes. The engagement of different biological responses downstream from $S1P_2$ signaling is amplified through activation of $G_{i/0}$, G_q and $G_{12/13}$. Nonetheless, $S1P_2$ has strongly been correlated with decreased migration, proliferation, angiogenesis and adhesion (Adada et al., 2013). It is well established that $S1P_2$ causes stress-fiber formation and disruption of adherens junctions, which leads to inhibition of endothelial cell migration. This response was found to be dependent on activation of Rho and Rho-associated protein kinase (ROCK) and subsequently inhibition of the PI3K pathway (Sanchez et al., 2007). It is worth to keep in mind that $S1P_2$ simultaneously inhibits Rac1 leading to anti-migratory responses (Arikawa et al., 2003). Moreover, macrophage migration is inhibited by the action of $S1P_2$ through enhanced cyclic adenosine monophosphate (cAMP) production and activation of protein kinase A (PKA) (Michaud et al., 2010). Several lines of evidence have associated $S1P_2$ with proliferation. In Wilm's tumor cells (WiT49 cells) S1P inhibits proliferation via $S1P_2$ (Li et al., 2008). The anti-proliferative action of $S1P_2$ is strengthened by results from animal and patient studies. Mice lacking $S1P_2$ exhibit diffuse large B-cell lymphomas, and patients with such symptoms were found to harbor a mutation in their $S1P_2$ gene. Thus, lack of $S1P_2$ or mutations in the $S1P_2$ gene result in enhanced cell growth, illustrating the inhibitory effects of $S1P_2$ on proliferation (Cattoretti et al., 2009).

2.3.2.3 $S1P_3$

$S1P_3$ can be found on the surface of virtually all types of cells, however, its expression is highest in the heart, lung and spleen. Functionally $S1P_3$ has been associated with the same kind of responses as $S1P_1$. $S1P_3$ activates $G_{i/0}$, G_q and $G_{12/3}$, and gives rise to cellular responses linked to proliferation, migration and angiogenesis. The final result of $S1P_3$ response is thus dependent on the cell type and which G protein is activated. $S1P_3$ has been closely associated with inflammation, and it has been suggested that it would function as a potential biomarker in lung injuries (Sun et al., 2012). The role of $S1P_3$ is also evident in MS, in which it together with $S1P_1$ is up-regulated in astrocytes causing inflammation in the brain. Thus, in MS, drugs targeting $S1P_3$ could be beneficial (Van Doorn et al., 2010). In contrast to its stimulatory effects, $S1P_3$ can act as an inhibitor of cellular events. $S1P_3$ has been correlated with anti-migratory and anti-proliferative effects during arterial injury. These notions originate from studies, in which defects in $S1P_3$ have resulted in an increased amount of smooth muscle cells in atherosclerotic lesions, pointing to an inhibitory role for $S1P_3$ (Keul et al., 2011). Furthermore, S1P by activating $S1P_3$ is able to decrease coronary blood flow (Murakami et al., 2010). Nonetheless, the function of $S1P_3$ is not restricted to normal cells $S1P_3$ plays an important role in cancers as well. In lung cancers, $S1P_3$ has been shown to collaborate with endothelial growth factor (EGF) receptor system to regulate proliferation and migration (Hsu et al., 2012) and in breast cancer $S1P_3$ in response to

estrogen is able to trans-activate EGF-receptor to regulate migration and proliferation (Sukocheva et al., 2006).

2.3.2.4 S1P$_4$

S1P$_4$ activates $G_{i/0}$ and $G_{12/13}$, albeit with a preference for $G_{i/0}$. The expression of S1P$_4$ is restricted to specific tissues such as lymphoid organs. As can be expected the knowledge we have gained about the nature and function of S1P$_4$ comes from studies mostly obtained from these tissues. Thus, to expand our understanding about the importance of S1P$_4$, more investigations need to be performed. Nevertheless, there are some reports showing both stimulatory and inhibitory responses triggered by this receptor. Overexpression of S1P$_4$ has been shown to have opposing effects in different cell types. In Chinese hamster ovary cells S1P$_4$ overexpression causes stress-fiber formation, hence, inhibition of migration coupled to $G_{i/0}$ and $G_{12/13}$, highlighting an inhibitory effect of S1P$_4$ (Graler et al., 2003). T-cells expressing S1P$_4$, but depleted of S1P1, lacked the capacity to migrate, suggesting S1P$_4$ to have no major importance for migration. However, results obtained from the same cells showed that S1P via S1P$_4$ was able to inhibit proliferation (Wang et al., 2005). In contrast to those results, the migration of Jurkat T cells was induced when S1P$_4$ was overexpressed, even in the absence of S1P (Graler et al., 2003). Thus, these reports suggest that the responses triggered by S1P$_4$ are cell type specific.

2.3.2.5 S1P$_5$

S1P$_5$ activates exclusively $G_{i/0}$ and $G_{12/13.}$ In a tissue screen study by Im and coworkers (2001) S1P$_5$ was found in a variety of different tissues including spleen, lung, placenta, aorta and peripheral blood leukocytes. However, high expression of S1P$_5$ can be mainly observed in the brain, where S1P$_5$ has been mostly studied. S1P$_5$ has been implicated in impeding migration of immature oligodendrocytes via Rho (Novgorodov et al., 2007), whereas survival of mature oligodendrocytes is stimulated by S1P$_5$ in a $G_{i/0}$ and AKT-dependent fashion. These results suggest that S1P$_5$ might function as a regulator of different developmental stages (Jaillard et al., 2005). Furthermore, S1P$_5$ has been demonstrated to play a fundamental role in maintaining the blood-brain barrier by decreasing permeability and increasing tight junctions. S1P$_5$ also decreases inflammation by inhibiting NF-kB activation and subsequent cytokine secretion as well as by decreasing transendothelial migration into brain parenchyma (Van Doorn et al., 2012).

Apart from its role in normal cell function, S1P$_5$ has also been shown to be involved in cancer cell behavior. Chang et al. (2009) reported that in prostate cancer cells S1P induced autophagy via S1P$_5$, demonstrating that this receptor plays an inhibitory role in proliferation and survival. In another study by Hu et al. (2010) in esophageal cancer cells overexpressing S1P$_5$, S1P-stimulation resulted in inhibition of proliferation and migration. Interestingly, S1P$_5$ overexpressing esophageal cancer cells were able to migrate more in the absence of S1P compared to cells expressing

empty vector. This observation led the authors to assume that esophageal cancer cells might in fact down-regulate $S1P_5$ to escape the inhibitory effect of $S1P_5$ (Hu et al., 2010). Collectively, based on the above-described results $S1P_5$ might function as an inhibitory receptor, giving rise to inhibition of cellular responses. However, to broaden our view more investigations dealing with the actions of this receptor must be performed. Equally important is the need for results from different cell types and animal studies.

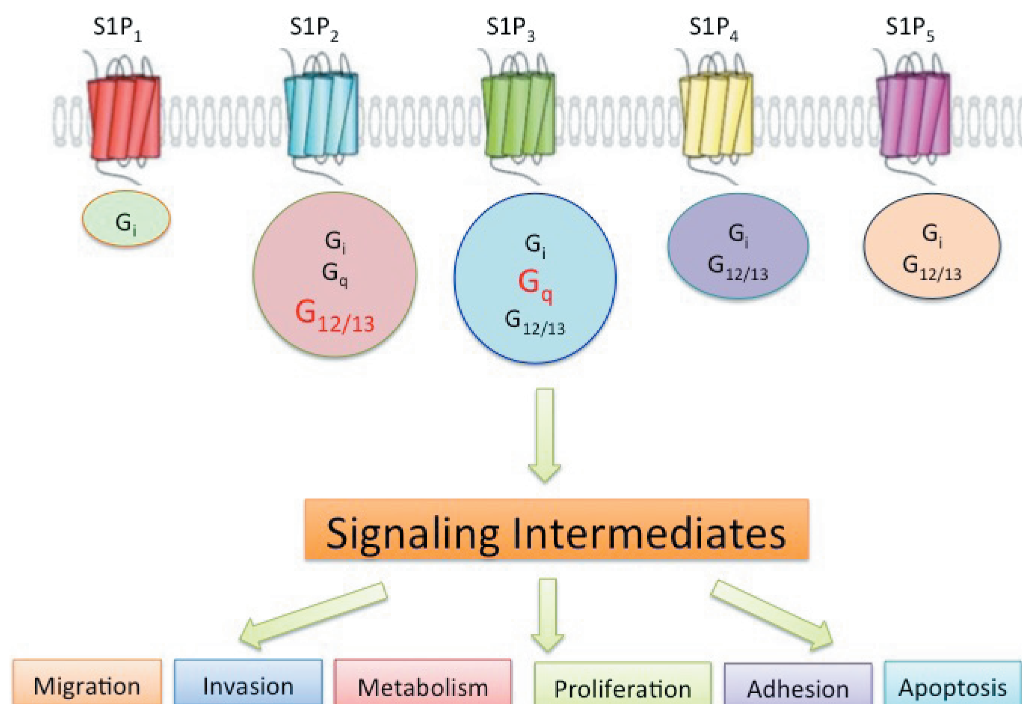

Figure 4. S1P-receptors and their downstream signaling events are depicted here. Depending on which receptor and G protein is activated, S1P stimulation elicits different cellular pathway responses. (Adapted and modified from Pyne & Pyne, 2013).

2.4 Sphingosine kinases in cancer

Two isoforms of sphingosine kinases termed SK1 and SK2 exist. Despite similarities in their structure, they exhibit differences in localization and function (Selvam & Ogretmen 2013).

SK1 is cytosolic and signals proliferation and migration. SK1 expression has been found to be up-regulated in many types of cancers correlating with an invasive phenotype of the cancer cells. However, no mutation in the SK1 gene has been found, suggesting that it is rather the SK1 activity that is enhanced during tumorigenesis. Many lines of evidence have implicated SK1 in tumor growth and metastasis, and in fact,

SK1 has been considered as an oncogene. As both high levels of S1P and elevated SK1 activity cause resistance to cytotoxic drugs and radiation, inhibition of SK1 has been proven beneficial in increasing the sensitivity of cancer cells to radiotherapy (Selvam & Ogretmen 2013; Newton et al., 2015). SK1 becomes activated via several signaling molecules. Protein kinase C (PKC) has been strongly associated with phosphorylation of SK1. PKC phosphorylates SK1 and triggers SK1 translocation to the plasma membrane. In addition, SK1 and VEGF have been shown to communicate, and this interplay might play an important role in cancer. VEGF is able to increase SK1 activity and SK1 is able to stimulate VEGF secretion, which of course is important in angiogenesis and vascularization (Tabasinezhad et al., 2014). VEGF-induced phosphorylation of SK1 triggers activation of RAS, ERK1/2-MAPK and subsequently cellular responses (Hait et al., 2006). There are several SK1 inhibitors, which have been shown to elicit excellent effects on tumor growth, angiogenesis and metastasis (Selvam & Ogretmen, 2013; Tabasinezhad et al., 2014).

SK2 is mainly found in the nucleus and cellular compartments and acts as a pro-apoptotic molecule. However, SK2 has recently been found to confer drug resistance, thus a role for SK2 in cancer is emerging. The SK2 inhibitor ABC294640 has been shown to inhibit growth and proliferation of many types of cancer cells, and to increase sensitivity to chemotherapy (Selvam & Ogretmen, 2013; Newton et al., 2015). *In vitro*, ABC294640 has been reported to induce autophagy and inhibits chemo-resistance mediated by NF-κB in breast cancer, thus ABC294640 has anti-estrogenic effects. Additionally, ABC294640 used with other inhibitors and drugs have shown additive effects on cancer cell proliferation. In fact, ABC294640 has reached phase 1 of clinical trials, where patients with advanced tumors are treated with this drug (Zu Heringdorf, 2013).

2.5 Hypoxia-inducible factors

Hypoxia-inducible factors (HIF) are a family of hypoxia inducible transcription factors consisting of three members termed HIF-1, HIF-2 and HIF-3, which are activated in response to low oxygen levels. HIFs are composed of two subunits: one alpha (α) and one beta (β) subunit. The HIFβ subunit (also called ARNT) is constitutively active, while the HIFα subunit is subject to regulation by different means (Ke & Costa, 2006). The HIF-1α and β subunits consist of several similar domains each with distinct function. The N-terminal domain functions as DNA-binding site, the central region (PAS 1 and 2) serves as hetero-dimerization domain and the C-terminus is used for recruitment of transcriptional co-activators. In addition to these domains, the alpha subunit contains an oxygen dependent degradation domain (ODDD), which plays an essential role in oxygen-dependent degradation of HIF-α (Ke & Costa, 2006; Ortmann et al., 2014). Figure 5 summarizes the structure of the HIF-1α subunit.

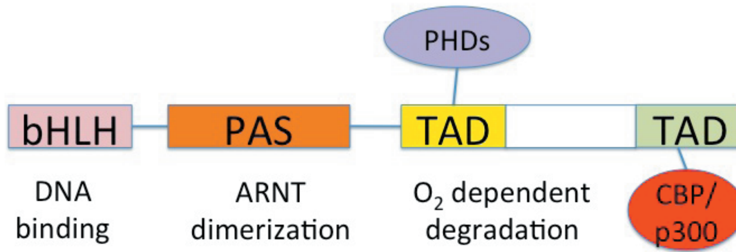

Figure 5. Schematic picture that shows the structure of HIF-1α with several domains. Each domain has a specific function. The bHLH domain within the N-terminus of the protein functions as DNA-binding site, the PAS domain is the site where HIF-1β (ARNT) binds. The TAD domain is the binding site for PHDs, a binding that triggers O_2 dependent degradation. The C-terminus TAD domain recruits co-activators (CBP/p300), which are important in target gene activation. (Adapted from Semenza, 2003).

2.5.1 HIF-1α

HIF-1α has been extensively studied for decades both *in vitro* and *in vivo* and it has been found that HIF-1α plays a major regulatory role both during development and normal physiology as well as in the pathology of a magnitude of different diseases including cancers. HIF-1α induces activation of a subset of different genes involved in migration and invasion, cell survival and growth, apoptosis, angiogenesis and metabolism. Thus, HIF-1α is a key player in many critical aspects of cancer biology.

One hallmark of cancer is increased energy consumption by cancer cells, which need increased glucose production and enhanced metabolism. Accumulating evidence suggests that HIF-1α is a modulator of metabolic processes by activating genes involved in metabolic pathways. HIF-1α is capable of switching oxidative metabolism to glycolytic metabolic reactions. HIF-1α increases glucose metabolism by enhancing the expression of Glut 1 and 3, and by enhancing the expression of metabolic enzymes such as hexokinases and phosphoglycerate kinase 1. Additionally, HIF-1α masterminds metabolic events by enhancing fatty acid synthesis and storage of triglycerides as well as reducing fatty acid oxidation. Furthermore, HIF-1α increases nutrition supply to cells by promoting angiogenesis. HIF-1α can also enhance metabolism under hypoxic conditions by increasing the activity of cytochrome c oxidase, which under low oxygen conditions can enhance electron transfer efficiency (Semenza, 2012a; Semenza, 2012b; Huang et al., 2014; Semenza, 2014).

To maintain a normal cell number in tissues, cells proliferate and undergo apoptosis, processes in which HIF-1α plays a fundamental role. Importantly, normal cell proliferation can be distinguished from abnormal cell growth by changes in the balance between proliferation and apoptosis. Neoplastic cells have the capacity to evade apoptosis and increase their proliferation rate. Traditionally, HIF-1α has been strongly associated with enhanced proliferation and survival of cancer cells by inducing numerous genes involved in these cellular events. HIF-1α induces transcription of a wide range of growth factors such as insulin like growth factor, transforming growth factor-α, vascular

endothelial growth factor, endothelin and erythropoietin. In addition to these, HIF-1α confers immortalizing effects on cancer cells by activating genes involved in telomerase maintenance (Semenza, 2012a; Semenza, 2014). Polymerases are a group of enzymes participating in the elongation of telomeres in DNA strands, hence, preventing senescent cells to enter apoptosis. In conditions low of oxygen, the transcription of the telomerase reverse transcriptase gene is enhanced by HIF-1α, resulting in increased cell proliferation (Semenza, 2014).

In a recent review, a complex trans-regulation between HIF-1α and cell cycle regulators has been discussed. HIF-1α has been implicated in regulating the cell cycle by affecting cell cycle regulators. Although contradictory to the traditional context that HIF-1α is an inducer of proliferation, HIF-1α has been shown to induce cell arrest in G1 phase by promoting the activity of p21 and p27, hence decreasing the activity of cyclin dependent kinases. On the other hand, cyclin dependent kinase 1 has been shown to phosphorylate HIF-1α on serine 668, which leads to activation of HIF-1α even in normoxia. This activation results in induction of target genes regulating cell proliferation and survival (Ortmann et al., 2014). Furthermore, on the one hand HIF-1α blocks the expression of the pro-apoptotic mitochondrial proteins (BAX and BID) and caspases, and on the other hand enhances the expression of anti-apoptotic proteins. In both cases these responses lead to decreased cell death (Semenza, 2012a). These findings suggest that HIF-1α both promotes and blocks proliferation. Thus, the role of HIF-1α in cell proliferation needs more studies in order to understand the exact mechanisms underlying how HIF-1α accomplishes these tasks.

HIF-1α has also been implicated in regulating angiogenesis and invasion by inducing genes involved in these cellular events. HIF-1α is able to transform cells from an epithelial to a mesenchymal phynotype. Both HIF-1α and its target genes have been shown to be up-regulated in many types of cancers, highlighting the important role and involvement of HIF-1α. Vascular endothelial growth factors (VEGFs), matrix metalloproteinases (MMPs), c-myc and RET are all well characterized genes regulating cancer cell migration and invasion. These genes have all been shown to be HIF-1α target genes. In addition to these, HIF-1α has been shown to activate the genes for lysyl oxidases LOX, LOXL1 and LOXL2, which are potent inducers of breast cancer cell migration and invasion (Semenza, 2003; Semenza, 2012a; Semenza, 2012b; Semenza, 2014).

HIF-1α has also been associated with chemoresistance by regulating genes involved in this event. The expression of multidrug transporters MDR1 and BCRP, which usually pump drugs out of the cell, is enhanced by HIF-1α. In addition, HIF-1α is capable of conferring resistance to DNA damages induced by chemotherapy by inhibiting the action of proteins of crucial importance in these events (Semenza, 2012a).

These findings verify HIF-1α to have pivotal role in regulating cellular events, in particular in cancer cells. The broad role of HIF-1α in cancer has made HIF-1α an attractive target for cancer therapy. Thus, several different inhibitors against HIF-1α have

been developed. The inhibitors target HIF-1α at the level of transcription, translation and activity. Many of these inhibitors have already reached clinical trials, and are tested on human patients with great success.

2.5.2 Classical regulation of HIF-1α

Like many other biological processes, also the HIF-system encounters endogenous inhibitors of its own. The main inhibitors are termed prolyl hydroxylases (PHDs) (Srinivas et al., 1999; Masson et al., 2001; Masson & Ratcliffe, 2003). However, there are two other enzymes involved in the regulation of HIF-1α, namely the acetyltransferase arrest-defective-1 (ARD1) (Jeong et al., 2002) and the factor inhibiting HIF (FIH) (Hewitson et al., 2002).

The main regulators of HIF-1α are PHDs, which in response to normoxia bind to the ODDD of the HIF-1α subunit, and hydroxylate it on proline 402 (Pro402) and 564 (Pro564). This hydroxylation targets HIF-1α for recognition by the von Hippel Lindau complex (pVHL), which polyubiquitinates and finally targets HIF-1α for degradation in the proteasome. However, under hypoxic conditions the activity of PHDs is blocked, preventing the binding of PHDs to the ODDD of the HIF-1α subunit, and consequently the destruction of it. Hence, HIF-1α becomes stabilized by hypoxia (Srinivas et al., 1999; Tanimoto et al., 2000; Masson et al., 2001; Masson & Ratcliffe, 2003). Upon stabilization, HIF-1α is translocated into the nucleus where it binds to its partner HIF-1β, forming the HIF-1 complex (Kallio et al., 1997). In the nucleus HIF-1 in association with co-partners such as CBP/p300 binds to hypoxia response elements (HRE) on a variety of different target genes of crucial importance both during normal development and in many types of disorders (Lando et al., 2002). The regulation of HIF-1α by PHDs is demonstrated in Figure 6.

ARD1 acts independently of oxygen as a facilitator of HIF-1α binding to the pVHL. ARD1 is able to acetylate HIF-1α at the Lys532 residue within the ODDD. This labeling results in de-stabilization of HIF-1α (Jeong et al., 2002). The importance of Lys532 residue has derived from studies where mutations of the Lys532 residue have been shown to stabilize HIF-1α (Tanomoto et al., 2000).

The recruitment of the co-partner CBP/p300 by HIF-1 involves hydroxylation of the asparagine 803-residue (Asn803) in the C-TAD domain of HIF-1α by FIH-1. High levels of oxygen can prevent the interaction between HIF-1 and CBP/p300 by blocking the activity of FIH-1, ultimately hampering the activation of HIF-1 target genes (Sang et al., 2002; Hewitson et al., 2002).

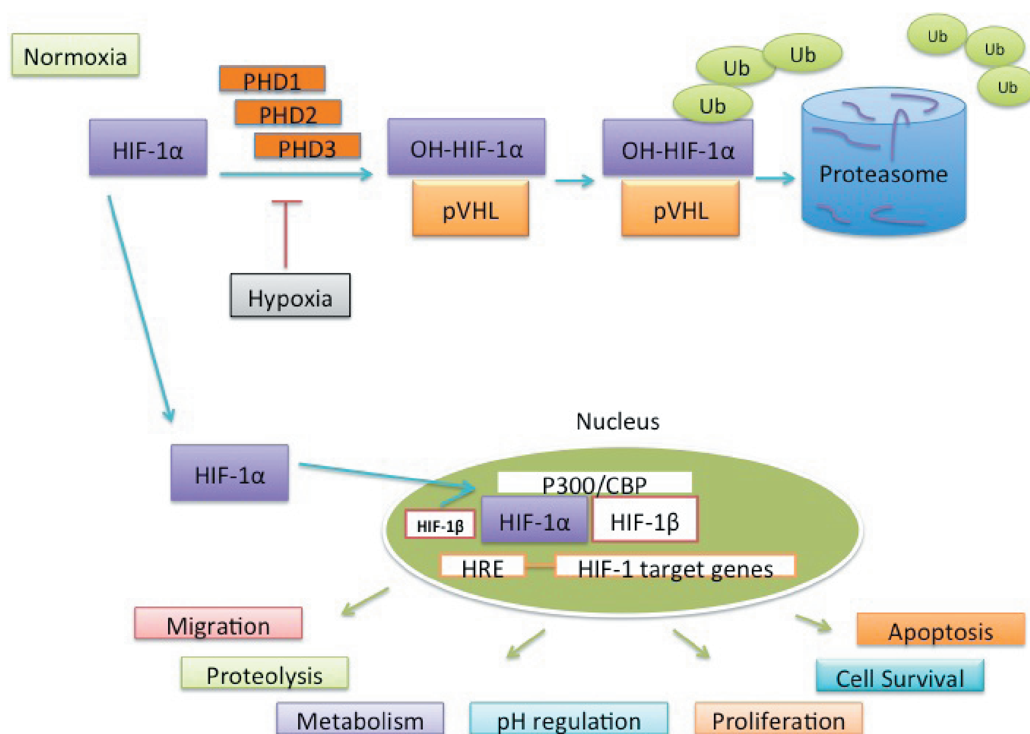

Figure 6. Normoxia induces activation of PHDs leading to binding of HIF-1α to pVHL and ubiquitination of HIF-1α and subsequent degradation of HIF-1α in the proteasome. Hypoxic conditions inhibit PHDs causing translocation of HIF-1α to the nucleus, where HIF-1α binds to HIF-1β forming the HIF-1 complex. Thereafter, HIF-1 recruits co-activators and binds to HRE-elements and activates target genes involved in many different cellular processes. (Adapted from Semenza, 2003).

2.5.3 Other modes of regulation of HIF-1α

During the past years, it has become more evident that HIF-1α can also be modulated in a non-hypoxic manner by the action of growth factors, cytokines as well as lipids. The major mode of regulation has been shown to be at the level of transcription and protein synthesis. MAPK and PI3K signaling pathways are the master regulators contributing to the enhancement of HIF-1α protein synthesis. Mammalian target of rapamycin (mTOR), p70S6K and the translation factor eIF4E are all crucial regulators of protein synthesis, of which all are involved in regulating HIF-1α protein synthesis (Semenza, 2003; Zhang et al., 2007). In addition, protein kinase C isoforms (PKCs) (Zhang et al., 2007) have been proven to be involved in regulating HIF-1α protein expression.

HIF-1α stability is subject to regulation by heat shock protein 90 (Hsp90) and the receptor for activated C kinase 1 (RACK1) in normoxia independently of pVHL. Hsp90 is a chaperone involved in protein folding and stabilization during stress and heat shock. Binding of HIF-1α to Hsp90 facilitates stability. RACK1 is a scaffolding protein with binding ability to a subset of different proteins, and is involved in stabilization of

proteins. When HIF-1α binds to RACK1 it is destined for degradation in the proteasome. Thus, Hsp90 and RACK1 function as competitors for HIF-1α binding and serve as regulator of HIF-1α depending on the circumstances and the preference of HIF-1α for these two molecules (Liu et al., 2007).

Moreover, a link between S1P, SK and HIF has emerged during the past few years. Initially, Anelli et al. (2008) showed that hypoxia, in fact, increased the expression and the activity of SK1, subsequently triggering up-regulation of S1P in U87MG glioma cells. The authors reported that inhibition of HIF-2α blocked SK1 and the production of S1P, whereas siRNA treatment of HIF-1α resulted in up-regulation of both HIF-2α and SK1. These changes in SK1 activity by hypoxia and HIFs correlated with enhanced proliferation and migration of glioma cells used in the study. In line with these results, Schwalm et al. (2008) was able to show that hypoxia up-regulates the expression and activity of SK1, and that HIF-1α and HIF-2α bind to the SK1 promotor region in the SK1 gene in endothelial cells to enhance migration of these cells. Furthermore, in vascular endothelial and smooth muscle cells, S1P via $S1P_2$ has been demonstrated to increase the expression and activity of HIF-1α in a pVHL-independent fashion (Michaud et al., 2009). Recently, Salama et al. (2015) demonstrated that in clear cell renal cell carcinoma, which is associated with mutations in VHL and increased HIF stability, the expression of SK1 is elevated. These elevations in the SK1 protein result in high levels of S1P and enhanced invasion of these cells.

2.5.4 HIF-2α

Although there are many structural and functional similarities between HIF-1α and HIF-2α, many studies have indicated that these two transcription factors, in fact, have opposing effects and that they activate a different subset of target genes. There are also differences in the expression pattern and the mode of activation of these two transcription factors. HIF-2α is more prone to become activated even under normoxia for a prolonged time, whereas at least normal hypoxic conditions are needed for HIF-1α for activation. However, when activated in normoxia, the duration time for HIF-1α activity is rather short (reviewed in Zhao et al., 2015b).

In mouse xenograft experiments, tumors originating from lung and colon tissues were shown to be exclusively dependent on the activity of HIF-1α for their growth, while HIF-2α was found to act as tumor suppressor (Imamura et al., 2009). In line with these results, Sun et al. (2013) demonstrated a negative role for HIF-2α in tumor growth in hepatocellular carcinoma cells (HCC). In this study, the authors reported that HIF-2α induced apoptosis. This apoptotic response of HIF-2α was dependent on the action of the transcription factor E2F1, which plays a role in controlling the cell cycle by inducing tumor suppressors such as p53. In contrast to these results showing an inhibitory effect of HIF-2α on proliferation, in $VHL^{-/-}$ mice the growth of tumors developing from kidney and liver were demonstrated to be HIF-2α dependent (Rankin et al., 2008). In accordance with the concept of HIF-2α regulating tumor growth and invasion, Zhao et al. (2015a) showed that human renal cancer tumorigenesis and

invasiveness are dependent on HIF-2α. In a review by Zhao et al. (2015b), several lines of evidence implicate HIF-2α as a key regulator of many aspects of tumor progression. HIF-2α activates genes important for metabolism, proliferation, angiogenesis and metastasis. Thus, HIF-2α might exhibit different effects on cellular events depending on the cell type and the genes it activates.

2.5.5 HIF-3α

Among the HIF-family members, HIF-3α is by far the least studied. Therefore, its function has remained elusive. There are, however, a few reports dealing with HIF-3α. Initially, it was demonstrated by Maynard et al. (2003) that several different splice variants of HIF-3α are direct targets for the pVHL ligase complex. This group even identified the proline residues in the ODD domain, and provided evidence that HIF-3α is degraded in a pVHL-dependent fashion similar to HIF-1α. Later on, HIF-3α was shown to act as an inhibitor of HIF-1α and HIF-2α in clear-cell renal cell carcinoma (CC-RCC), thus, suppressing tumor growth and metastasis (Maynard et al., 2007). Another report by Ando et al. (2013) is providing insights on different splice variants of HIF-3α, where the HIF-3α4 variant acts as a negative regulator of angiogenesis in malignant meningiomas. Recently published data has, however, shown that HIF-3α is also a hypoxia inducible transcription factor inducing genes important for cellular function (Zhang et al., 2014b). Thus, more studies are needed to broaden our understanding about the role of this transcription factor. However, these few results provide exiting insights of HIF-3α, and offer new opportunities for research findings and future intervention strategies.

2.6 Extracellular degrading enzymes

The extracellular matrix (ECM) is a scaffold of several different proteins surrounding tissues in an extremely organized way. In order to be able to migrate and invade, cells need to have the ECM degraded. The degradation of the basement membrane and the extracellular matrix is governed by diverse mechanisms and several factors play major roles in this process. The most studied enzymes with the ability to brake down the components of the ECM are matrix metalloproteinases (MMP) (Stamenkovic, 2000; Sternlicht & Werb, 2001; Löffek et al., 2011; Yadav et al., 2014), lysyl oxidases (LOX) (Mayorca-Guiliani & Erler, 2013), urokinase plasminogen activator and its receptor (uPA and uPAR) (Noh et al., 2013), matriptase (Miller & List, 2013) and kallikreins (Romero Otero et al., 2014). Figure 7 shows the major ECM-degrading enzymes.

The LOX family contains five members termed LOX and LOX-like (LOXL) 1-4 and they are widely distributed in the tissues. LOX is secreted as zymogen and activated through cleavage of the glycosylated N-terminal domain of LOX by bone morphogenetic protein 1 (BMP1). LOX functions as a collagen and elastin cross-linker, hence, modifying the extracellular matrix. As LOX is a HIF target gene and often up-regulated by hypoxic conditions, it has gained a substantial amount of interest

in the tumor biology filed. LOXs have been implicated in a variety of different cellular responses, including migration, invasion and angiogenesis (Mayorca-Guiliani & Erler, 2013).

Kallikreins are secreted serine proteases widely expressed in the tissues. The human kallikrein family comprises of 15 members, representing up to date the largest serine protease family. Kallikreins are synthesized as prekallikreins, which need to be activated in order to be able to exert their effects on the ECM. The activation of kallikreins is facilitated by factor XII. This same enzyme is also able to generate plasmin from plasminogen. The main function of kallikreins is to cleave kininogen to release kallidin. Kallikreins are implicated in several physiological and pathophysiological conditions. They are involved in regulating blood pressure, vascular cell growth and smooth muscle contraction. Kallikreins are also involved in neurodegenerative diseases. Nonetheless, kallikreins have also been linked to cancers including ovarian and prostate cancer. Due to their wide expression pattern, kallikreins function as biomarkers for prostate cancer (Romero Otero et al., 2014).

The uPA receptor is a glycosylphosphatidylinositol (GPI)-anchored protein linked to the plasma membrane. The binding of pro-uPA to uPAR results in cleavage of plasmin from plasminogen. Plasmin is able to activate MMPs, a response leading to degradation of ECM. In cancers, the uPA-uPAR system has been shown to be up-regulated and play a fundamental role in regulating many central processes important in the progression of cancers. It is well documented that uPAR is also a HIF-1α target gene. Hence, uPAR is up-regulated in hypoxia and modulates cancer cell proliferation, growth, migration and invasion. uPAR is able to interact with other receptor systems, for example growth factor receptors as well as integrin receptors, which all elicit important roles in invasion and metastasis. Thus, inhibition of uPA-uPAR has been of great interest in treatment of cancer patients (Noh et al., 2013).

Matriptase is a type II transmembrane protease that activates the GPI-anchored prostatin, which initiates a proteolytic cascade important for the degradation of ECM. Similar to many other biological systems, the matriptase-prostatin system has its own endogenous inhibitor, namely hepatocyte growth factor activator inhibitor-1 (HAI-1). The activation of matriptase encounters sequential cleavages and close collaboration with its inhibitor. In fact, many studies have shown that matriptase is co-expressed with HAI-1. Known *in vivo* substrates of matriptase include hepatocyte growth factor (HGF) and prostatin, while *in vitro* matriptase cleaves uPA, the G-protein-coupled protease activated receptor-2 (PAR2), the platelet-derived growth factor-D (PDGF-D) and the human acid-sensing ion channel 1. Abnormalities in matriptase activity have been liked to several diseases including human autosomal recessive icthyosis with hypotrichosis (ARIH), Netherton's syndrome and cancers. Recently, matriptase has emerged as a potential oncogene playing a central role in cancer progression. It has been found to be up-regulated in many types of cancers governing many central events occurring during proliferation and migration (Miller & List, 2013).

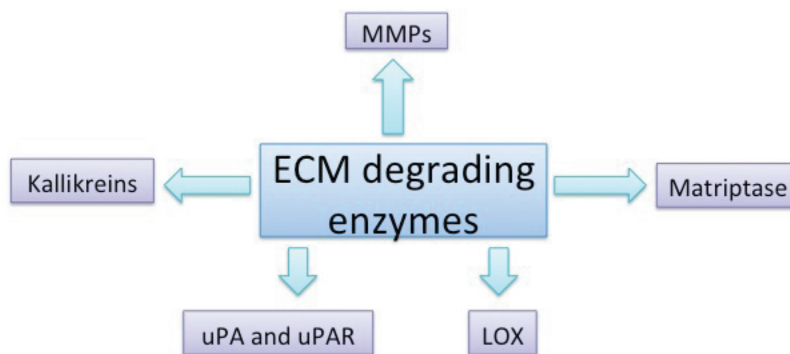

Figure 7. Demonstration of several different ECM-degrading enzymes of crucial importance for migration and invasion of cancer cells.

2.6.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc dependent proteolytic enzymes involved in the degradation of the extracellular matrix. The MMP family is huge and is divided into several subgroups depending on their function and localization (shown in Figure 8). Up to date 26 members of MMP family have been characterized in humans. MMPs can be secreted into the extracellular milieu, be membrane bound via a GPI-anchor or reside in the plasma membrane as transmembrane proteins. The MMP family consists of gelatinases, metalloelastases, enamelysins, membrane-type MMPs, collagenases, stromelysins, tissue inhibitors and others. In the upcoming sections only the gelatinases and MT1-MMP will be described. A brief description of TIMPs will also be provided (Stamenkovic, 2000; Sternlicht & Werb, 2001; Löffek et al., 2011; Yadav et al., 2014).

The structure of MMPs is conserved between different members of the MMP family, and they share common domains. There are three main domains: the pro-peptide domain, the catalytic domain, and the haemopexin-like C-terminal domain. MMPs are synthesized as zymogens with a pro-peptide. Upon activation the pro-peptide is cleaved resulting in a shift towards a smaller molecular weight of the entire protein. The catalytic domain has a conserved sequence, where zinc ions can bind. Thus, this domain is the catalytically active domain. The haemopexin-like C-terminal domain is used for interaction with other proteins (Stamenkovic, 2000; Sternlicht & Werb, 2001; Löffek et al., 2011; Yadav et al., 2014).

Due to their ability to cleave a wide spectrum of substrates, MMPs have been found to have profound and essential roles in many physiological and pathophysiological processes. Thus, they regulate events during tissue remodeling and bone growth and they take crucial part in angiogenesis and vascular development. MMPs are also involved in immune response, wound healing as well as migration. Interestingly, MMPs have been shown to perform apoptotic actions as well by cleaving FAS ligand and activating TGF-β. In contrast to that, overproduction of MMPs induces tumor progression and

metastasis (Stamenkovic, 2000; Sternlicht & Werb, 2001; Löffek et al., 2011; Yadav et al., 2014; Itoh 2015).

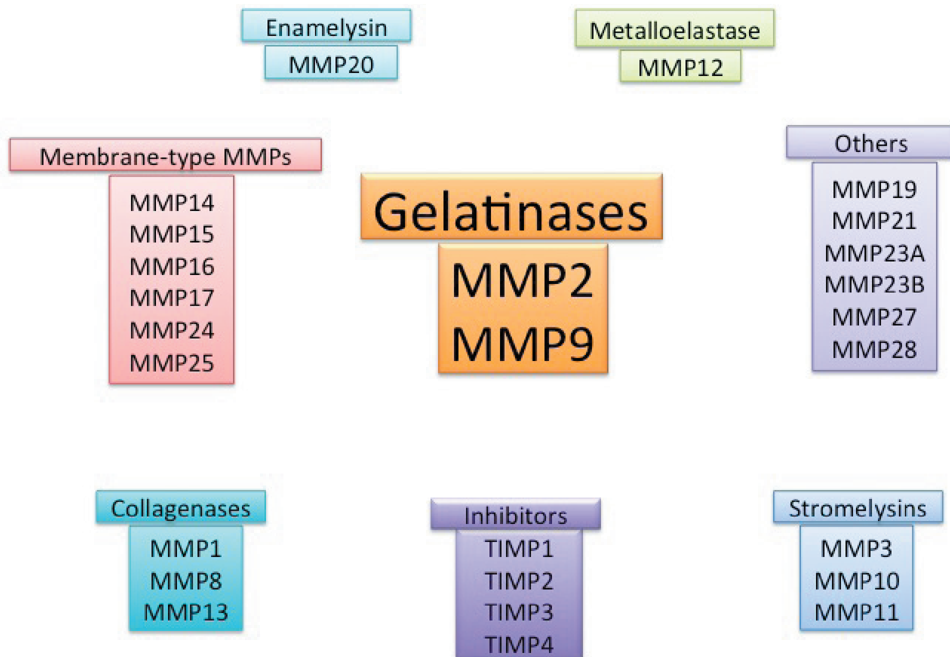

Figure 8. Presentation of the MMP family. The gelatinases MMP2 and MMP9, which were studied in detail in this thesis, are placed in the center. (Adapted and modified from https://upload.wikimedia.org/wikipedia/commons/2/22/Matrix_Metalloproteinases.png)

2.6.2 Regulation of MMPs

Decades of studies and substantial amount of evidence have established new research strategies and broadened our understanding of the connection between carcinogenesis and MMPs. In fact, MMPs have been demonstrated to be altered and to play major roles in several types of cancers (Stamenkovic, 2000). Therefore, understanding the mechanistic regulations of these endopeptidases is of crucial importance in establishing new therapeutic interventions.

MMPs are regulated at the level of gene transcription, extracellular localization and compartmentalization, proenzyme activation, as well as by inhibition of enzymatic activity. In respect to gene regulation, MMPs are regulated by a variety of different means. Transcriptional and post-translational regulations by growth factors, cytokines, micro-RNA (miRNA), as well as direct binding to the AP-1 binding site in the promoter region of the MMP gene are the most common mechanisms of regulation (Stamenkovic, 2000; Löffek et al., 2011; Yadav et al., 2014).

As most MMPs are secreted into the extracellular space, the catalytic activity of these is already determined by their localization in the tissue. The activation of the

proenzyme requires removal of the pro-peptide. The removal of the pro-peptide is achieved by direct removal by the aid of other endo-peptidases, conformational changes in the pro-domain or by chemical alternations (Löffek et al., 2011; Yadav et al., 2014).

Within the MMP family a subgroup of four members exist. These are termed tissue inhibitors of matrix metalloproteinases (TIMPs). TIMPs function as endogenous inhibitors of MMPs. Additionally, α_2-macroglobulin the broad-spectrum inhibitor of proteinases, has been implicated in inhibiting MMPs. The inhibition of MMPs requires interactions between the endogenous inhibitors and activators. This is well documented for activation of MMP2 by TIMP2 and MT1-MMP (Löffek et al., 2011; Yadav et al., 2014).

2.6.3 MT1-MMP

Membrane-type MMPs are a subfamily of MMPs consistent of 6 members, of which MT1-MMP (also called MMP14) is the most studied and quite well understood. Membrane-type MMPs reside in the plasma membrane, of which some are transmembrane proteins, while a couple of them are connected to the plasma membrane via a GPI-anchor. MT1-MMP is widely expressed in several different tissues. Nonetheless, it is overexpressed in many types of cancer cells and its functions were therefore mainly discovered in cancer cells. It participates in proteolysing a wide range of ECM components, including collagen (Itoh, 2015). MT1-MMP is a key player in migration and invasion. Thus, due to these particular functions it has gained a substantial amount of interest. In respect to cancers, MT1-MMP is responsible for many aspects characteristic for tumor progression ranging from simple cell motility to deadly metastatic features. MT1-MMP is a crucial component of several membrane structures involved in cell motility and migration such as focal adhesions, filopodia, lamellipodia, invadopodia and pseudopodia. In these structures MT1-MMP associates with proteins important for migration such as focal adhesion kinase and Rac1 (Itoh, 2015). Nonetheless, MT1-MMP can also promote carcinogenesis by a non-proteolytic mechanism. It has been demonstrated to modulate the expression and activity of several proteins regulating cell motility and invasion such as HIF, Notch and Rho-A. Due to its broad involvement in tumorigenesis, MT1-MMP has become one of the most targeted ECM-degrading enzymes in the treatment of cancers, and it is an extremely attractive molecule for future therapeutic interventions (Itoh, 2015).

2.6.4 Gelatinases: MMP2 and MMP9

Matrix metalloproteinases 2 and 9 are secreted into the extracellular space. They are called gelatinases due to their ability to use gelatin as substrate. MMP2 and MMP9 are among the most widely distributed MMPs and are involved in the breakdown of collagen IV. Gelatinases participate in a subset of different cellular functions including proliferation, survival, angiogenesis as well as migration and invasion (Stamenkovic 2000; Sternlicht & Werb 2001; Löffek et al., 2011; Yadav et al., 2014). Although secreted into the extracellular space, MMP2 is known to interact with other membrane

proteins such as integrins (α4β3) in the outer leaflet of the membrane, and degrade the extracellular matrix without any need for secretion. Similarly, it has been suggested that MMP2 can interact with its activator, MT1-MMP in the membrane, thereby regulating invasion (Sternlicht & Werb, 2001; Mitra et al., 2003).

2.6.5 Sphingolipids and MMPs

There is a substantial amount of evidence from *in vitro* studies suggesting that S1P can regulate MMP2 and MMP9 both in normal and cancer cells. In endothelial cells, S1P has been suggested to induce MMP2 and VEGF via the transcription factor ZNF580 promoting proliferation, migration and angiogenesis of the cells (Sun et al., 2010). Moreover, Chang et al. (2013) demonstrated that S1P enhances the expression of VEGF-C via a MMP2 and FGF-dependent signaling pathway in human umbilical vein endothelial (HUVEC) cells. Furthermore, in ovarian cancer cells S1P enhances invasion by activating MMP2 through Gi and Rac1 (Devine et al., 2008). In breast cancer MCF10A cells, S1P induces secretion of MMP9 and migration via $S1P_3$ and G_q (Kim et al., 2011). In accordance with these results, the invasion of head and neck squamous cell carcinomas has been associated with enhanced activity of SK1, MMP2 and MMP9 as well as activation of the ERK1/2 and /AKT pathway (Tamashiro et al., 2014).

Many previous reports have elucidated a role for S1P and also lysophosphatidic acid (LPA) in MT1-MMP regulation and the importance of MT1-MMP in migration and invasion (Gingras et al., 2008, Sun et al., 2010, Wu et al., 2005, Annabi et al., 2009; Devine et al., 2008). Interesting results have shown that in hepatocellular carcinoma cells lysophosphatidic acid (LPA) induces invasion by activating MMP9 (Lou et al., 2013). Furthermore, LPA induces invasion of MCF-7 cells, which is mediated by RhoA, ROCK, MMP2 and MMP9 (Sun et al., 2015). Annabi et al., (2009) showed that S1P increases MMP2 activity and migration of CD133(+) glioblastoma stem cells, and that siRNA knock down of MT1-MMP abolishes S1P-induced cell migration.

Thus, accumulating evidence suggests that there is a connection between sphingolipids and MMPs of essential importance in progression of cancers. Due to characteristic features of S1P and MMPs, inhibition of S1P receptors could indeed be beneficial for cancer patients.

2.6.6 Calpain

Calpains are a widely expressed family of intracellular calcium-dependent proteolytic enzymes responsible for the cleavage of a wide range of different proteins. The calpain family consists of 15 members, however, calpain 1 and 2 (mu-calpain and m-calpain respectively) are the most thoroughly studied. As many other proteins, calpains also encounter their own endogenous inhibitor termed calpastatin. Calpains have been implicated in several central events both during development and in many diseases, including many types of cancers. Giving the nature of cancer cell properties, calpains have been linked to all features and events of cancer cell transformation due to their wide spectrum of substrates (Franco & Huttenlocher, 2005; Leloup & Wells, 2011; Moretti et al., 2014).

First identified by Carraghare and coworkers, calpains were shown to be involved in cell transformation due to the fact that their expression and activity was induced by v-Src, v-Myc, k-Ras,v-Fos and v-Jun (Carragher et al., 2002; Carragher et al., 2004). Likewise, Niapour et al. (2008) showed that c-Myc activates calpains by suppressing their inhibitor calpastatin. Thus, all these events highlight the role of calpains in malignant transformation.

Calpains are known to have a central role in regulating motility, migration and invasion of cancer cells by enhancing the expression and activity of a wide range of different proteins important for these processes. In respect to motility, both calpain 1 and calpain 2 play a central role. Calpain 1 is important for the formation of focal adhesion complexes in the front of the cell, while in the rare of the cell calpain 2 is essential for the disassembly of the complexes, hence, for cell retraction (Glading et al., 2002). Furthermore, calpains have been implicated in modulating migration by proteolysing the tumor suppressor E-cadherin, thereby facilitating migration of prostate cancer cells (Rios-Doria et al., 2003). In addition, calpains have been well established in regulating invasion by modulating the expression, secretion and activity of MMPs in several types of cancer cells. Chen et al. (2013) showed that in hepatocellular carcinoma cells siRNA silencing of calpain 1 and 2 resulted in a decrease of both MMP2 and MMP9 secretion, which reflected the invasive phenotype of these cells. Furthermore, the migration and invasion of lung cancer cells have been linked to increased calpain activity and MMP9 expression (Meng et al., 2009). Jang and co-workers (2010) reported that the invasion of glioblastoma cells was shown to be dependent on calpain 2 and MMP2. Likewise, the invasion of osteosarcoma cells was suggested to be dependent on calpain 1 and 2 and MMP2 (Fan et al., 2009). Furthermore, the invasion of T-antigen-immortalized mouse embryonic fibroblasts cells into matrigel has been shown to be dependent on calpain and MMP2 expression (Postovit et al., 2002). In addition to these results, calpains have been implicated in regulation of cellular processes by modulating MT1-MMP. Endothelial sprout formation and invasion have been shown to be dependent on calpain and MT1-MMP in 3D collagen matrices (Kang et al., 2011; Kwak et al., 2012). Thus, all these data in the literature highlight the broad spectrum of regulation of cellular processes by calpains, and the down-stream signaling pathways they activate to achieve their tasks.

2.7 Studies related to thyroid cancer

2.7.1 MMPs and HIF in thyroid cancer

A growing body of evidence about HIF and MMPs suggests that they have a role in thyroid cancer. Several previous studies have shown that an increased expression and activity of both MMP2 and MMP9 correlate with an enhanced invasion of thyroid cancer cells (Rajoria et al., 2011; Zhang et al., 2012: Yang et al., 2013). Moreover, MT1-MMP has also been associated with an invasive phenotype of thyroid cancer cells (De Amicis et al., 2013). Burrows et al. (2010) demonstrated that in normal thyroid tissues HIF-1α is barely detectable. However, the authors were able to detect high amounts of HIF-1α in

different types of thyroid cancer cells, correlating with high grade of invasiveness. The highly expressed HIF-1α protein correlated with the activity of PI3K and RAF/MEK/ERK1/2 signaling pathways. A role of HIF-1 α and MMP9 is also evident in papillary K1 thyroid cancer cells. Tan and co-workers (2014) showed that curcumin has anti-migratory effects on K1 cells. This response was due to the ability of curcumin to decrease the mRNA and protein levels of HIF-1α as well as attenuate the binding capacity of HIF-1α to the HRE-elements on target genes. In addition, curcumin inhibited the expression of MMP9, resulting in inhibition of migration of the cells. Two parallel studies by Wang and collaborators (2013a; 2013b) demonstrated that high expression of HIF-1α and HIF-2α in papillary thyroid cancer cells is highly associated with advanced TNM stage, reflecting the involvement of HIF-1α/2α in thyroid malignancies. Furthermore, Rajora et al. (2014) could show a link between estrogen and HIF in HUVEC cells, in a way that HIF-1α inhibition was able to attenuate estrogen-induced migration of these cells. Collectively, a role for HIF and MMPs in thyroid cancer progression is emerging, which is of great importance considering the future medical interventions.

2.7.2 S1P in thyroid cancer

The significance of S1P and its receptors for thyroid cancer proliferation and migration has been one of the milestones of Törnquist laboratory. In previous studies from Törnquist laboratory, follicular ML-1 and FTC-133 cells as well as the anaplastic C643 and THJ-16T cells were used. The receptors dominating in ML-1 and FTC-133 cells are $S1P_{1,3}$, and the main receptor regulating cell function in C643 is $S1P_2$. Not surprisingly and as can be expected, S1P acts as a potent inducer of migration and invasion of ML-1 and FTC-133 cells (Balthasar et al., 2006; Balthasar et al., 2008; Bergelin et al., 2009; Bergelin et al., 2010; Törnquist 2013), whereas the migration of C643 and THJ-16T cells is blocked by S1P (Asghar et al., 2012). These differences in the S1P response highlight the importance of receptor expression profile and the ultimate response of S1P being exclusively dependent of the receptors present in the cells. The existence of communication between the S1P receptor system and the receptors for growth factors is well established. In line with this concept, it was demonstrated that a complex crosstalk between S1P receptors and vascular endothelial growth factor receptor 2 (VEGFR2) exists. VEGF can modulate S1P receptors and SK1 activity, and S1P is simultaneously able to influence the expression and activity of VEGFR2 as well as the secretion of VEGF-A. Furthermore, it was demonstrated that PI3K, ERK1/2, PKCα are important intermediates of this interplay, which subsequently leads to enhanced migration of ML-1 cells (Balthasar et al. 2008; Bergelin et al., 2009; Bergelin et al. 2010). In addition to these findings, the Törnquist laboratory has also been able to present a link between SK1 and VEGFR2 in thyroid cancer. SK1 (Guan et al., 2001) and VEGFR2 (Turner et al., 2003; Kim et al., 2006) have been shown to be up-regulated in thyroid cancers, associated with a high grade of invasiveness. Thus, accumulating evidence suggests that S1P and VEGFR2 are emerging as potent regulators of thyroid cancer cell migration and invasion. S1P and SK1 have also been shown to play key roles in calcium signaling in thyroid

cancer. S1P is able to modulate calcium channels and signaling events downstream from the channels important for proliferation and migration (Törnquist, 2012).

2.7.3 Treatment of thyroid cancer and challenges

The overall prognosis for some types of thyroid cancers is relatively good and favorable when the diagnosis is carried out early enough at a proper stage of the disease. Currently, there are several types of treatments for thyroid cancers. The methods, however, are, as for any other type of cancer, highly traditional and unspecific (Schneider & Chen, 2013; Jin et al., 2013). Normally, partial or total thyroidectomy is carried out followed by radiation and chemotherapy. However, the method of treatment should be chosen with accurate consideration depending on the type of the cancer and the stage of the tumor. One of the hallmarks of thyroid cancer is the recurrence of the disease after the first line of treatment. Recurrences can occur both locally at the site of the original tumor, however, metastases can also emerge leading to management difficulties in the second line of treatment (Schneider & Chen, 2013; Jin et al., 2013). The currently available chemical inhibitors used for thyroid cancer treatment are inhibitors of the genes altered in these groups of diseases. Often, the inhibitors target MAPK/ERK and VEGFR2, influencing cellular growth and metastasis. Nevertheless, these inhibitors are shown to be ineffective, highlighting the importance of more efficient, specific and targeted drugs, increasing the survival of the patients (Jin et al., 2013; Perri et al., 2014).

As S1P is a potent inducer of thyroid cancer cell migration and invasion, S1P-receptors and SK1 could serve as good and attractive candidates for cancer therapy. In fact, targeting these has been proven to be beneficial in clinical trials. Currently, there are several S1P-receptor modulating drugs in advance phases of clinical trials. The pharmaceuticals are aimed to target $S1P_{1,3}$ to block invasion, angiogenesis and proliferation of cancer cells. At the same time, molecules that enhance $S1P_2$ function are acquiring increased attention. Thus, activation of $S1P_2$, which normally is an anti-migratory receptor, might be beneficial for cancer patients (Zu Heringdorf et al., 2013)

2.7.4 FTY720, S1P-receptor modulating drug

Fingolimod (FTY720) is an immunosuppressive drug approved by FDA and EMA for treatment of multiple sclerosis (MS). FTY720 inhibits egress of lymphocytes from lymph nodes by inhibiting S1P1, thus preventing the migration of immune cells to the brain. Due to these actions, FTY720 is used in the treatment of MS (Matloubian et al., 2004; Lo et al., 2005; Chung & Hartung, 2010; Bigaud et al., 2014; Kim et al., 2015). It is an agonist for all S1P-receptors except for S1P2. However, it has highest affinity for S1P1, but affects also S1P3 very strongly. Exogenous FTY720 is taken up by cells and is phosphorylated by SK2 (Paugh et al., 2003), after which p-FTY720 is secreted by cells into the extracellular space, where it binds to S1P-receptors. The inhibitory effect of FTY720 on S1P-receptors is due to its ability to internalize the receptors, and cause degradation of S1P-receptors in the proteasome (Oo et al., 2007).

In addition to its immunosuppressive effects, FTY720 displays promising effects on cancer cell migration and proliferation as it has been shown to act as an inhibitor of SK1. FTY720 has also been shown to inhibit proliferation of a variety of different cancer cells by triggering G1 arrest and up-regulating p21 and p27. Furthermore, the anti-proliferative effect of FTY720 is due to its ability to increase the expression of PTEN, and to subsequently inhibit AKT. Moreover, FTY720 has the capacity to block the mouse double minute 2 homolog (MDM2), which is an E3 ubiquitin ligase and a negative regulator of p53. Therefore, as can be expected, FTY720 enhances the stability of p53 causing increased apoptosis and decreased growth. Furthermore, FTY720 has the ability to induce activation of caspase-3 and subsequently apoptosis. In addition to its anti-proliferative effects, FTY720 displays anti-migratory capacities as well. FTY720 achieves this by inhibiting integrins and activating RhoA (Pyne & Pyne, 2013, Kunkel et al., 2013 and Selvam & Ogretmen, 2013; Alshaker, 2013). Thus, FTY720 has a broad spectrum of effects on cancer cells.

3. AIMS

The role of sphingolipids in thyroid cancer has remained poorly understood. Previous investigations by our group, however, demonstrated that S1P very potently induces migration of thyroid follicular ML-1 cancer cells via $S1P_{1,3}$ and $G_{i/0}$ (Balthasar et al., 2006). Moreover, we showed that S1P receptors crosstalk with VEGFR2, which is of crucial importance for ML-1 cell migration. The aim of this study was to further investigate the mechanisms underlying the S1P-evoked migration and invasion of ML-1 cells. For this purpose we studied specifically:

1. The significance of HIF-1α for S1P-induced migration of thyroid cancer cells.
2. The role of MMP2 and MMP9 in S1P-evoked invasion of thyroid cancer cells.
3. The effect of the S1P-receptor modulator Fingolimod (FTY720) on the proliferation and invasion of thyroid cancer cells.

4. MATERIALS AND METHODS

4.1 Cell lines

In this project several thyroid cell lines were used, which were cultured according to **Table 1** and maintained in a humidified incubator at 37C˚ and under 5% CO_2. For experiments in hypoxia, cells were grown in a hypoxia workstation with 1% oxygen at 37 ˚C.

Table 1. The maintenance and the composition of culture medium for different cell lines.

Cells	**Medium**
ML-1 (I, II, III) and C643 cells (III)	Cultured in DMEM (Dulbecco´s modified Eagle's medium) supplemented with 2 mM L-glutamine, 10% FCS and 100 U/ml penicillin-streptomycin
FTC-133 cells (I, II, III)	Grown in DMEM + Ham's F12 medium (1:1) supplemented with 10% FCS and 2 mM L-glutamine
Nthy-ori 3-1 CELLS (III)	Cultured in RPMI 1640 medium supplemented with 2 mM Glutamine, 10% FBS, and 1% penicillin/streptomycin

4.2 Whole cell lysates (I, II, III)

After being treated as indicated, cells were washed with ice-cold PBS, and lysed in lysis buffer (10 mM Tris pH 7.7, 150 mM NaCl, 7 mM EDTA, 0.5% NP-40, 0.2 mM PMSF, 0.5 µg/ml leupeptin). Cell lysates were centrifuged at 13 000 rpm for 15 min at 4°C, whereafter pellets were removed and supernatants were collected. Protein concentrations were determined with the BCA Protein Assay kit (bicinchoninic acid), thereafter Laemmli sample buffer (LSB) was added and the lysates were boiled to be used for PAGE-analyses.

4.3 Immunoprecipitation (I)

Cells were treated as indicated and lysed with IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.2 mM PMSF, 0.5 µg/ml leupeptin). Protein concentrations were determined as described earlier, and lysates with equal protein concentrations and volumes were pre-cleaned with 20 µl of Protein A/G PLUS-agarose beads for 1 h at 4°C. Pre-cleaned lysates were incubated with 2 µg of antibody or IgG control overnight at 4°C followed by incubation with 40 µl of Protein A/G PLUS-agarose beads for 2 h at 4°C. Thereafter, lysates were centrifuged at 13 000 rpm for 2 min at 4°C. After discarding the supernatants, the agarose beads were washed with IP washing buffer (50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% NP-40), and Laemmli sample buffer (LSB) was added, the samples were boiled and run on SDS-PAGE.

4.4 SDS-PAGE and western blotting (I, II, III)

Equal amounts of protein were loaded on SDS-PAGE gradient gel (8-12% polyacrylamide) and later proteins were transferred onto a nitrocellulose membrane by electrophoresis. Thereafter, membranes were blocked with 5% milk in TBST (Tris-buffered saline (NaCl, 150 mM; Tris-base, 20 mM (pH 7.5)) containing 0.1% Tween 20 for 1 h at room temperature followed by incubation in primary antibody overnight at 4°C and in horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Proteins were detected by incubating membranes with Plus ECL solution. Protein bands were analyzed with the ImageJ program. As a loading control Hsc70 or β-actin was used and target protein densitometry was normalized with the loading control. Protein amounts in collected medium were normalized to total protein concentrations in respective samples.

4.5 Cell migration (I, II, III)

One day prior to the experiment the upper surface of Transwell inserts (6.5 mm-diameter and 8 µm pore size) were coated with 5 µg/cm2 human type IV collagen. Cells were serum-starved overnight. At the day of the experiments, the inserts were reconstituted with medium and the cells were treated with inhibitors as indicated. After being detached, 200 000 cells in serum-free medium were added into the upper wells of the inserts. Chemoattractants were added into the lower wells. The inhibitors were added to both wells. The cells were allowed to migrate as indicated. After removing the un-migrated cells from the upper surface of the membrane with a cotton swab, migrated cells were fixed with 2% paraformaldehyde in PBS for 10 min and stained with 0.1% crystal violet in 20% methanol for 5 min. Thereafter, migrated cells were counted in a light microscope.

4.6 Transfection with siRNA (I, II)

Cells were transfected with siRNA or plasmids with either the N-TER, HiPerfect transfection reagents or by electroporation with Amaxa Cell Line Optimization Nucleofector Kit and Amaxa electroporation device according to the manufacturer's instruction. Transfected cells were plated for 24 h and serum starved for another 24 h. Then, cells were treated and the experiments were carried out as indicated.

4.7 RNA extraction and reverse transcriptase PCR (I)

For extraction of total RNA the Aurum Total RNA Mini kit was used and RNA concentrations were determined using the RiboGreen RNA Quantitation Reagent according to the manufacturer's instructions. Thereafter, Reverse transcriptase PCR was

performed with SuperScript III Reverse Transcriptase to produce cDNA according to the manufacturer's instructions.

4.8 Quantitative real-time PCR (I)

The quantitative PCR assays were designed using the Universal ProbeLibrary Assay Design Center (www.roche-applied-science.com). Reaction mixtures were prepared with ABsolute QPCR Rox Mix or with the KAPA Probe Fast qPCR Kit and real-time quantitative PCR was performed using the Applied Biosystems 7900HT Fast Sequence Detection System or the StepOnePlus Real-Time PCR system. The amplification results were analyzed with the SDS and RQ Manager programs. GAPDH and HPRT1 were used as reference genes.

4.9 Luciferase assay (I)

To determine the luciferase activity of HIF-1 cells were co-transfected with either TK-Luc and Ubi-Renilla plasmid or HRE-Luc and Ubi-Renilla plasmid by electroporation with an Amaxa nucleofector device. Cells were lipid-starved 24 h after transfection, and the next day treated as indicated. Luminescence was measured with the DualGlo Luciferase Assay System according to the manufacturer's instructions. Renilla luciferase luminescence was used to normalize Firefly luciferase luminescence.

4.10 Zymography (II, III)

Cells were treated as indicated, medium was collected and equal volumes of sample medium were mixed with loading buffer (0.1 M Tris-phosphate buffer (pH 6.8) containing 20 % glycerol, 6 % SDS, 0.04 % bromphenol blue), and electrophoresed on 10% (SDS)-polyacrylamide gels containing 1mg/ml gelatin. First, gels were incubated in zymo buffer **I** for 30 min. Thereafter, gels were incubated for 30 min in the zymo buffer **II**. To allow gelatinolytic activity gels were incubated in zymo buffer **III** overnight at 37 °C. Then the gels were stained with Coomassie Brilliant Blue R250 for 1-2 h, and de-stained for 1-2h. Gelatinolytic activity was quantified by the ImageJ software. The data was normalized with the respective total protein concentrations of the samples.

Table 2. The composition of zymo buffers.

Buffer	**Composition**
zymo buffer **I**	50 mM Tris-HCl containing 2.5 % Tween 80 and 0.02 NaN_3 (pH 7.5)
zymo buffer **II**	50 mM Tris-HCl containing 2.5 % Tween 80 and 0.02 NaN_3, 1 µM $ZnCl_2$ and 5 mM $CaCl_2$ (pH 7.5)
zymo buffer **III**	50 mM Tris-HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$ and 0.02% NaN_3 (pH 7.5)

4.11 Calpain activity assay (II)

The activity of calpains was determined using a calpain activity assay according to the manufacturer's instructions. Two million cells were plated on 10 cm plates and treated with 100 nM S1P for the indicated times. Then, cells were harvested and lysed, and protein concentrations were determined as described earlier. The fluorometric readings were measured using a Victor fluorescence analyzer at excitation of 400 and emission of 505 nm, directly proportional to calpain activity and the values were normalized with the respective total protein concentrations in the plates.

4.12 Proliferation assay (I, II, III)

4.12.1 Cell Titer

Totally, 10 000 cells/well were plated on 96-well plates and serum starved for 24 h prior to treatment. Thereafter cells were treated as indicated and the experiments were terminated by using CellTiter® cell proliferation assay kit according to the manufacturer's instructions. The colorimetric reactions were measured by reading absorbance at an excitation of 492 nm and emission at 505 nm using a Victor fluorescence analyzer.

4.12.2 ^{3}H-thymidine incorporation assay (I, III)

To further measure the proliferation, ^{3}H-thymidine incorporation assay was performed. For the assay 75,000 cells were cultured for 24 h on 35 mm plates and serum starved for 24 h. Thereafter, cells were treated as indicated, followed by incubation with ^{3}H-thymidine (0.4 µCi/ml) for at least 4 h. Then, the cells were washed with ice cold PBS. After that the cells were incubated with 5% trichloroacetic acid for 10 min followed by incubation with 0.1 N NaOH for another 10 min. The radioactivity directly proportional to cell proliferation was measured by using a Wallac 1410 liquid scintillation counter.

4.13 Fluorescence-activated cell sorting

For the assay, cells were grown in 10 cm plates, and complete medium was changed to SFM overnight. The cells were then treated as indicated, washed with ice-cold PBS and detached. Next, 500 000 cells were suspended and incubated in propidium iodide (PI) solution (0.05 mg/ml PI, 3.8 µM sodium citrate and 0.1% Triton-X-100 in PBS) for 15 min at room temperature. Analyses of the samples were performed by flow cytometry using ModFit LT 4.1 software (Verity Software House Inc., Topsham, ME, USA)

5. RESULTS AND DISCUSSION

5.1 HIF-1α participates in S1P-evoked migration (I)

5.1.1 S1P induces HIF-1α expression and activity

The role of S1P in regulating migration of ML-1 follicular thyroid cancer cells had previously been thoroughly investigated by our group, and it was known that S1P strongly induces migration of these cells via $S1P_{1,3}$ and $G_{i/0}$ proteins (Balthasar et al., 2006). Furthermore, HIF-1α was shown to be a potent inducer of cancer cell migration and tumor growth as well as angiogenesis (Semenza, 2003; Semenza, 2010). These facts suggested that HIF-1α might be involved in S1P-induced migration of thyroid cancer cells. It turned out that S1P up-regulated HIF-1α protein expression in a time- and concentration dependent fashion under normoxic conditions in both ML-1 and FTC-133 cells. In addition, the expression of HIF-1α in hypoxia was studied, and results showed a substantial increase of HIF-1α protein expression in ML-1 cells. The effect of S1P on HIF-1α activity was also studied, and S1P was able to increase the activity of the HIF-1. To find out target genes, siRNA against some known classical HIF-1-target genes of importance for migration was used. It was found that S1P-induced gene expression of VEGF-A (vascular endothelial growth factor), AMF (autocrine motility factor) and TGFα (transforming growth factor) (Semenza, 2003) was abolished when HIF-1 gene was silenced by siRNA, indicating that these are HIF-1α target genes in ML-1 cells. As it was shown that S1P enhanced HIF-1α, it was of interest to find out the mediators of this response. For this purpose some signaling intermediates known to be of importance for S1P-signaling in ML-1 cells and also in regulating HIF-1α were investigated. It was found that S1P enhanced HIF-1α via $S1P_3$, $G_{i/0}$ and the downstream effectors MEK, PI3K and PKCβI and mTOR in ML-1 cells. In contrast, PKCα did not participate in the S1P-evoked regulation of HIF-1α expression. Collectively, S1P enhanced the expression and activity of HIF-1α via PKCβI, PI3K, ERK1/2, and induced genes important for migration.

5.1.2 S1P increases translation and stability of HIF-1α

Since S1P enhanced the protein expression of HIF-1α, it was of interest to figure out if S1P achieved this by inducing transcription. However, it was observed that the S1P-induced HIF-1α protein is not due to enhanced transcription, which raised the speculation that S1P might affect the translation or stability of HIF-1α. According to Semenza (2003) HIF-1α protein synthesis is regulated by p70S6K and eIF4E, therefor, it was interesting to find out if S1P was increasing the synthesis of HIF-1α protein. As expected, S1P phosphorylated eIF-4E on Ser209, 4EBP1 on Ser65 and p70S6K on Thr389, and inhibition of these could abolish the S1P-evoked increase in HIF-1α protein levels, meaning that S1P induced protein synthesis of HIF-1α in ML-1 cells. The same

kinds of responses were also observed in FTC-133 cells. These results were in line with previously published data (Zhang et al., 2007) showing that HIF-1α accumulation was mediated by classical PKC isoforms as well as phosphorylation of Akt, ERK, 4E-BP1 and p70S6K in lung cancer cells.

In addition, hypoxia is known to increase the stability of HIF-1α by inhibiting the activity of PHDs and hence the interaction between HIF-1α and the pVHL complex (Semenza 2003). Thus, the possibility of S1P having an effect on the stability of HIF-1α was investigated. By performing chase-experiments with cycloheximide (CHX) and study its effect on the S1P, $CoCl_2$ and hypoxia induced HIF-1α, it was found that S1P, in fact, also increased the stability of HIF-1α. This response turned out to be independent of pVHL, since S1P-induced HIF-1α was still bound to pVHL in pull-down experiments even though S1P decreased the hydroxylation of HIF-1α on pro402. S1P had, however, no effect on pro506 residue. Both Hsp90 and RACK1 were previously shown to participate in stabilization of HIF-1α. Binding to Hsp90 stabilizes HIF-1α, while HIF-1α is destined for degradation when it binds to RACK1 (Liu et al., 2007). Thus, it was asked if Hsp90 and RACK1 could have a role in regulating HIF-1α. Inhibition of Hsp90 did indeed prevent S1P from inducing HIF-1α protein, however, any changes in binding of HIF-1α to Hsp90 could not be observed due to technical difficulties. Taken together, S1P might increase both the translation and stability of HIF-1α, albeit the S1P-induced stability is not dependent on VHL.

5.1.3 HIF-1α is involved in basal and S1P-induced ML-1 cell migration

Since S1P treatment increased HIF-1α expression in both ML-1 and FTC-133 cells, and many previously published data showed that HIF-1α is important for migration (Semenza, 2003) it was interesting to explore the role of HIF-1α in S1P-induced migration. For this purpose both small molecular inhibitors and siRNA against HIF-1α were used, and it was found that inhibition of HIF-1α in both ML-1 and FTC-133 cells with small molecular inhibitors could abolish S1P-induced cell migration. However, results from siRNA experiments showed that silencing of HIF-1α could decrease basal migration. Interestingly, siRNA against HIF-1α was not able to totally abolish the S1P-induced migration, suggesting that there might be other players in S1P-regulated migration. Migration experiments were also performed under hypoxic conditions, as hypoxia is known to induce migration by up-regulating HIF-1α. Surprisingly, neither basal migration nor S1P-induced migration could be induced in hypoxia. It was hypothesized that hypoxia might inhibit unknown factors important for migration and S1P-signaling, explaining the responses observed. Taken together, it can be concluded that S1P up-regulates HIF-1α by increasing both stability and protein synthesis. Furthermore, HIF-1α regulates basal ML-1 migration and, in part, S1P-induced migration. Thus, inhibition of HIF-1α might serve as a favorable therapeutic approach for thyroid cancer patients.

5.2 MMP2 and MMP9 are involved in S1P-induced invasion of ML-1 cells (II)

5.2.1 The effect of S1P on the secretion, activity and expression of MMP2 and MMP9

Matrix metalloproteinases are degrading enzymes of the extracellular matrix facilitating cell migration and invasion of several types of cancer cells (Folgueras et al., 2004). Especially MMP2 and MMP9 have been shown to regulate migration and invasion of ML-1 cells (Rajoria et al., 2011). It was earlier shown that S1P is a potent inducer of ML-1 cell migration (Balthasar et al., 2006) leading to the speculation that MMP2 and MMP9 might be of importance for S1P-induced invasion of ML-1 cells. The results showed that S1P caused a rapid increase of both MMP2 and MMP9 secretion and activity. However, long incubation of cells with S1P (48 h) resulted in an increase only in MMP2 expression. In addition, results showed that S1P modulated MMP2 and MMP9 via $S1P_{1,3}$. These results were in line with previously published data that showed S1P to enhance the secretion, activity and expression of MMP2 and MMP9 (Devine et al., 2008; Sun et al., 2010; Kim et al., 2011;). Thus, S1P evokes expression, secretion and activity of MMP2 and 9 in ML-1 cells.

5.2.2 MMP2 and MMP9 participate in S1P-evoked invasion of ML-1 cells

As we had previously shown that S1P strongly induces migration of ML-1 cells (Balthasar et al., 2006), and that S1P enhances MMP2 and MMP9 secretion and activity, it was asked whether these two MMPs are mediators of S1P-evoked invasion. Both pharmacological inhibitors and siRNA against MMP2 and MMP9 were used. The inhibitors were able to totally abolish S1P-induced invasion. The results from siRNA experiments, however, showed that despite a substantial down-regulation of the expression and activity of MMP2 and MMP9, S1P could not be completely prevented from inducing invasion. This led to the assumption that other ECM-degrading enzymes might be involved in the S1P-evoked invasion of ML-1 cells. As the MMP family has many other members (Stamenkovic, 2000), the effect of S1P on MMP1, MMP3, MMP8 and MMP13 was tested. S1P had either no effect on the secretion or expression of these, or these proteins could not be detected in our investigations. In addition to MMPs, there are many other ECM-degrading enzymes that facilitate migration and invasion. The secretion of kallikreins (Romero Otero et al., 2014) and uPA (Noh et al., 2013) were investigated, however, these were not detected in the collected medium. The effect of S1P on the expression of matriptase (Miller & List 2013), LOX (Mayorca-Guiliani & Erler, 2013), and uPAR (Noh et al., 2013) were also studied, however, S1P was without any effect on these enzymes. Taken together, MMP2 and MMP9 participate in ML-1 cell invasion evoked by S1P, albeit there might also be other players, yet to be identified, in this regulation.

5.2.3 Calpains and Rac1 mediate S1P-induced secretion of MMP2 and MMP9

Previous findings showed that calpains are involved in cleaving several different proteins (Storr et al., 2011) including MMPs (Kang et al., 2011; Kwak et al., 2012). Furthermore,

S1P was demonstrated to activate calpains in human hepatocarcinoma cells (Chen et al., 2013). These observations raised interests to investigate the effect of S1P on calpains, and the involvement of calpains in both invasion and MMP regulation. Evidence could be provided that S1P could rapidly increase the activity of calpains time-dependently. Inhibition of calpains by ALLN resulted in both attenuation of invasion and secretion of MMP2 and MMP9. Rac1 (Ras-related C3 botulinum toxin substrate 1) is involved in migration and invasion by forming lamellipodia in the rare side of a migrating cell (Murali & Rajalingam, 2014). Previously, it was shown that Rac1 mediated the S1P-induced migration of ML-1 cells (Balthasar et al., 2006). In addition Rac1 was shown by Devine et al. (2008) to be involved in S1P-evoked regulation of MMPs. Based on these findings, we speculated that Rac1 could potentially be a mediator of the S1P-effect on MMPs in ML-1 cells. As expected, inhibition of Rac1 attenuated the S1P-evoked MMP2 and MMP9 secretion. In conclusion, calpains and Rac1 participate in S1P-evoked MMP2 and MMP9 secretion and invasion.

5.3 FTY720 (Fingolimod) attenuates thyroid cancer cell invasion and proliferation (III)

5.3.1 FTY720 modulates S1P-receptors

FTY720 is a S1P receptor modulating drug, which has been shown to cause internalization and degradation of $S1P_{1,3}$ (Oo et al., 2007; Mousseau et al., 2012). As expected, in ML-1 cells FTY720 evoked down-regulation of $S1P_1$ in a time- and concentration dependent manner. However, the expression of $S1P_3$ was inhibited only by high concentrations of FTY720. The FTY720-triggered down-regulation of $S1P_1$ was due to lysosomal degradation. In contrast to our results, Oo et al. (2007) demonstrated that FTY720 caused proteasomal degradation of $S1PR_{1/3}$ in HUVEC and embryonic kidney 293 cells. Interestingly, it was found that $S1P_1$ in untreated cells was degraded in the proteasome, pointing to a role for the proteasome in the fine-tuning of cellular levels of $S1P_1$ protein. Taken together, FTY720 induces lysosomal degradation of $S1P_1$, while under normal conditions $S1P_1$ is degraded in the proteasome. Moreover, high concentrations of FTY720 are needed in order to be able to down-regulate $S1P_3$, perhaps due to the fact that FTY720 might have a weaker binding affinity for $S1P_3$.

5.3.2 FTY720 inhibits invasion of thyroid cancer cells

It was previously shown that S1P and SK1 strongly evoked migration and invasion of ML-1 cells via $S1P_{1,3}$ (Balthasar et al., 2006; Bergelin et al., 2009). Therefore, the role of FTY720 in migration of thyroid cells was investigated, and results showed that FTY720 attenuated invasion of several thyroid cancer cell lines, but was without an effect in normal thyroid cells. In addition, the S1P-induced invasion of both ML-1 and FTC-133 cells was significantly attenuated by FTY720. These results are strengthened by previously published data, which demonstrated that FTY720 inhibits migration of cancerous cells (Tonelli et al., 2010; Lim et al., 2011; Li et al., 2012; Mousseau et

al., 2012). Moreover, p-FTY720 could attenuate S1P-induced invasion of ML-1 cells. Previously it was shown that S1P induced invasion of ML-1 cells through VEGFR2, ERK1/2, PKCα and βI and HIF-1α (Balthasar et al., 2006, Balthasar et al., 2008). Accordingly, FTY720 inhibited the expression of VEGFR2, PKCα and -βI. However, FTY720 was not able to significantly decrease the expression of HIF-1α. Furthermore, FTY720 inhibited MMP2/9 activity and abolished S1P-induced MMP2/9 secretion and ERK1/2 phosphorylation in ML-1 cells. Surprisingly, FTY720 was not able to affect the expression of SK1, p70S6K or mTOR, all signaling intermediates inhibited by FTY720 in other cancer cell types (Tonelli et al., 2010; Zhang et al., 2010). Taken together, FTY720 blocked migration by inhibiting signaling intermediates important for thyroid cancer cell migration. However, the final outcome of the FTY720 treatment depends on the concentration of the drug and the cancer cell type it is administered to, suggesting the importance of cancer cell specificity.

5.3.3 FTY720 attenuates proliferation of thyroid cancer cells

Different concentrations of FTY720 were evaluated on the proliferation of thyroid cells and could show that FTY720 inhibited proliferation of all cell lines tested in a concentration dependent fashion. To further investigate the underlying mechanism behind this inhibition, ML-1 and FTC-133 cells were used, and it was found that FTY720 arrested cells in G1 phase and decreased the amount of cells in the S phase of the cell cycle in both cell lines. In ML-1 cells, the FTY720-evoked G1 arrest was due to an up-regulation of p21 and p27. These results were in accordance with previously published data by Permpongkosol et al. (2002), who demonstrated that FTY720 blocked proliferation of human prostate carcinoma DU145 cells by inducing apoptosis, and arresting cells in the G1 phase of the cell cycle through induction of p21. Combination therapy with several different drugs in treating cancer is emerging as a powerful tool to enhance the efficiency of the drugs. Therefore, cells were treated with FTY720 or p-FTY720 in combination with doxorubicin. However, it was not possible to see any additive or synergetic effects. Collectively, FTY720 acts as a blocker of thyroid cancer cell proliferation.

6. CONCLUSIONS

Based on the results from these three studies, we conclude that:

1. HIF-1α is a mediator of S1P-induced migration in ML-1 and FTC-133 cells.
2. MMP2 and MMP9 participate in S1P-evoked invasion.
3. FTY720 could function as a promising inhibitor of thyroid cancer cell proliferation and invasion, by blocking signaling intermediates important for these processes.

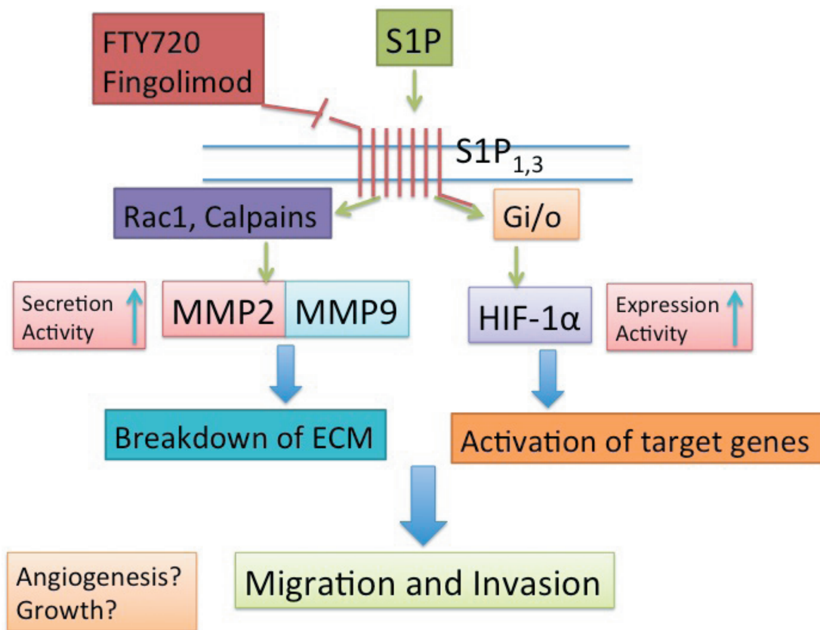

Figure 9. Schematic picture, demonstrating the involvement of HIF-1α and MMP2/9 in S1P-induced pathology of follicular ML-1 thyroid cancer cells.

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