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SPHINGOSINE 1-PHOSPHATE RECEPTOR 2
AND THE TRPC1 ION CHANNEL AS
REGULATORS OF HUMAN THYROID
CANCER CELL MIGRATION AND
PROLIFERATION



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To my family

ABSTRACT

Thyroid cancer is the most common endocrine cancer and currently there is no effective treatment available against the most aggressive and highly invasive forms of thyroid cancer. Sphingosine 1-phosphate (S1P) is a bioactive lipid which binds to S1P1-5 receptors and activates different G-proteins and their associated downstream signaling pathways. S1P receptors and Transient receptor potential canonical (TRPC) calcium channels are ubiquitously expressed in human tissues and regulate many cellular processes including cell migration and proliferation.

In the first study, we showed that the S1P1-3 are expressed in the anaplastic thyroid cancer (ATC) cell line C643. S1P attenuated the invasion and migration in these cells by binding to S1P2, which strongly couples to G12/13 protein and activates the Rho and ROCK pathway. In addition, we detected the expression of a non-conducting form of the ether a'-go-go related gene (HERG) potassium channel protein and characterized the significance of this channel in the regulation of ATC C643 cell migration. We found that S1P transiently decreased the expression of HERG protein. Thus, the S1P evoked attenuation of migration in these cells is, in part, due to the decreased expression of the HERG potassium channel protein.

The identity of TRPC channels in human thyroid is unknown. In the second study, we investigated the expression of transient receptor potential canonical (TRPC) channels in human normal thyroid and follicular thyroid cancer (FTC) ML-1 cells, and described the function of TRPC channels in ML-1 cell migration and proliferation. We reported that several TRPC isoforms, including TRPC1, are expressed in these cells. To investigate the importance of TRPC1 in human thyroid cancer, we generated stable TRPC1 knock-down cells (TRPC1-KD). We showed that in the ML-1 cells, TRPC1 functions as a receptor operated calcium entry channel (ROCE), but not as a store operated calcium entry channel (SOCE). In addition, the invasion, migration and proliferation were attenuated in TRPC1-KD cells. An interesting finding here was that the pro-migratory receptors S1P3 and the vascular endothelial growth factor receptor 2 (VEGFR2) were down-regulated in the TRPC1-KD cells, and we could rescue this effect by restoring TRPC1 in TRPC1-KD ML-1 cells. Thus, the effect on receptor expression was mediated through a calcium-dependent mechanism mediated by TRPC1 channel activity. In addition, the expression of the downstream entities including PKC β , PKC δ and ERK1/2, which are involved in S1P3 evoked expression of VEGFR2 and hypoxia-inducible factor 1-alpha (HIF1 α) were downregulated in TRPC1-KD cells. Furthermore, the secretion and activity of matrix metalloproteinase 2 and 9 (MMP2 and MMP9) was attenuated in TRPC1-KD cells. These results highlight the importance of TRPC1 channels in thyroid cancer cell migration and proliferation.

The extracellular matrix (ECM) is a highly dynamic and complex assembly of numerous structural and functional proteins. After acquiring a migratory phenotype, cancer cells secrete matrix metalloproteinases (MMPs), which break down the ECM (mainly collagen) and facilitates invasion. In the last study, we found a novel mechanism by which S1P, through activation of S1P2, can regulate cancer cell invasion. In the ATC C643 cells, S1P attenuated the expression, secretion and activity of MMP2 and to a lesser extent MMP9. The inhibitory effect on at least MMP2 is mediated through the Rho and ROCK pathways. In addition, S1P attenuated the activity of calpain, resulting in decreased secretion of active MMP2 and MMP9.

Taken together, the information presented in this thesis can be of significance when designing novel strategies to curtail aggressive and metastatic thyroid cancer and possibly cancers in general.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following research articles, which are referred to in the text by their roman numerals.

- I. Sphingosine 1-phosphate and human ether-a'-go-go-related gene potassium channels modulate migration in human anaplastic thyroid cancer cells.
Muhammad Yasir Asghar, Viitanen Tero, Kempainen Kati and Törnquist Kid. 2012 *Endocrine-related cancer*, 19 667-680. doi: 10.1530/ERC-12-0092.

- II. Transient Receptor Potential Canonical (TRPC1) Channels as Regulators of Sphingolipid and VEGFR Receptor Expression: IMPLICATIONS FOR THYROID CANCER CELL MIGRATION AND PROLIFERATION.
Muhammad Yasir Asghar, Magnusson Melissa, Kempainen Kati, Sukumaran Pramod, Löf Christoffer, Pulli Ilari, Kalhori Veronica and Törnquist Kid. 2015 *The Journal of Biological Chemistry*, 290 (26) 16116-16131. doi: 10.1074/jbcM115.643668.

- III. Sphingosine 1-phosphate attenuates MMP2 and MMP9 in thyroid cancer cells: Importance of S1P₂.
Muhammad Yasir Asghar, Kempainen Kati and Törnquist Kid. 2017, *Under Revision*.

ABBREVIATIONS

AC	Adenylate cyclase
Akt	Protein Kinase B
ATC	Anaplastic thyroid cancer
CaM	Calmodulin
CNS	Central nervous system
CVS	Cardiovascular system
DAG	Diacylglycerol
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FTC	Follicular thyroid cancer
GPCR	G protein coupled receptor
HERG	Human ether a'-go-go related gene
HIF	Hypoxia-inducible factor
IP3	Inositol 1,4,5-triphosphate
Kv	Voltage dependent potassium channel
MAPK	Mitogen-activated protein kinase
MMP2	Matrix metalloproteinase 2
MMP9	Matrix metalloproteinase 9
MMPs	Matrix metalloproteinases
MTC	Medullary thyroid cancer
PKC	Protein kinase C
PLC	Phospholipase C

PTC	Papillary thyroid cancer
PTH	Parathyroid hormone
Rac	Ras-related C3 botulinum toxin substrate
Rho	Ras homolog gene family member
ROCE	Receptor operated calcium entry
ROCK	Rho-associated kinase
S1P	Sphingosine 1-phosphate
S1P1	Sphingosine 1-phosphate receptor 1
S1P2	Sphingosine 1-phosphate receptor 2
S1P3	Sphingosine 1-phosphate receptor 3
S1P4	Sphingosine 1-phosphate receptor 4
S1P5	Sphingosine 1-phosphate receptor 5
SK	Sphingosine kinase
SOCE	Store operated calcium entry
SERCA	Sarco/endoplasmic reticulum calcium -ATPase
T3	triiodothyronine
T4	Thyroxine
TSH	Thyroid stimulating hormone
TRP	Transient Receptor Potential
TRPC	Transient Receptor Potential Canonical
VEGFR2	Vascular endothelial growth factor receptor

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1. INTRODUCTION

Thyroid cancer is the most prevalent endocrine cancer. The number of new diagnosed cases is increasing worldwide (Finnish Cancer Registry, Cancer Statistics at www.cancerregistry.fi, (Siegel et al. 2016)). Usually the prognosis is quite good. However, due to mutations or dedifferentiation of thyroid cells, highly aggressive and metastatic forms of thyroid cancer may arise. The active metastasis to bones, brain and lungs is common which leads to no survival and the patients die within a few months. Currently, there is no effective treatment available for such aggressive thyroid tumors (Nagaiah et al. 2011; Nikiforova et al. 2003; Smallridge et al. 2009). However, recent advances in genome-wide sequencing analysis have presented some novel therapeutic strategies to treat anaplastic thyroid cancer (X. Liu et al. 2013; Wang et al. 2014).

Sphingolipids have been initially thought to have only a structural role in cell membranes. Later, it was found that these lipids are involved in signal transduction and control a multitude of cellular responses. One of the metabolites of sphingolipid metabolism is sphingosine 1-phosphate (S1P), which is a bioactive lipid and regulates several cellular processes including cell migration and proliferation (Pyne & Pyne 2010). S1P is present in the blood and lymph and can act on distinct tissue sites. S1P is synthesized within the cell and transported out of the cell where it acts as an autocrine or paracrine signal. S1P binds to S1P1-5 receptors and activates specific G-proteins and through the activation of specific downstream signaling cascade, modulates cell migration and proliferation of both normal and cancer cells (Spiegel et al. 2002). S1P1 and S1P3 activate the PI3K-Akt and Rac pathway, which results in an enhanced invasive potential and migration of the cancer cells (Ishii et al. 2002). On the contrary, S1P2 through G12/13 activates the Rho and ROCK pathway and attenuates invasion and migration (Takuwa et al. 2011; Witt et al. 2012).

Increasing evidence during the past decade indicates that the ion channels are expressed in many cancer forms in humans and regulate a multitude of cellular processes, including migration and proliferation (Prevarskaya et al. 2010). Hence, ion channels have emerged as potential targets for cancer therapy. In this thesis, we have investigated the importance of HERG potassium channels and the transient receptor potential canonical (TRPC) calcium channels in cell migration and proliferation.

Kv 11.1 (HERG) potassium channels are 6 transmembrane voltage-gated ion channels, and belong to the ether a'go-go related (EAG) family of potassium channels. Previous reports have established that HERG channel expression is upregulated in several types of cancer cells, such as in brain cancer, blood cancer, stomach cancer, colon cancer, breast cancer, and skin cancer. HERG channels have been shown to regulate cell migration and proliferation (D'Amico et al. 2013). However, in thyroid cancer, the expression and significance of the HERG potassium channel remained unknown.

Calcium signaling is of crucial importance in the regulation of many cellular processes. Calcium channels gate calcium ions (Ca^{2+}) into the cell. After entry into a cell, calcium binds to calcium-binding proteins and activates downstream signaling pathways to end in specific cellular responses. In many cancer forms, calcium signaling plays an essential role in cell migration and proliferation through transient receptor potential canonical (TRPC) channels, a subfamily of the transient receptor potential (TRP) superfamily of calcium ion channels. The TRPC subfamily comprises six members in humans, TRPC1 and TRPC3-7. TRPC1 is ubiquitously expressed in human tissues and has been shown to regulate cell migration and proliferation. TRPC1 functions both as a receptor operated calcium entry (ROCE) and a store operated calcium entry (SOCE) channel (Prevarskaya et al. 2010; Riccio et al. 2002; Abramowitz & Birnbaumer 2009). However, the TRPCs expression in human thyroid remained elusive.

The transformation of a simple epithelial cell to a highly malignant tumor cell is a long and complex cascade of events. The cells first reside and grow in a primary tumor, then acquire the ability to move by changing their shape from epithelial to mesenchymal. They also release enzymes, mainly matrix metalloproteinases (MMPs), to degrade the extracellular matrix, which facilitates cancer cell invasion and metastasis (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011). Previously, S1P has been shown to induce invasion and migration of cancer cells by activating the small GTPase Rac and associated secretion and activity of MMP2 and MMP9 (Devine et al. 2008; Kalhori & Törnquist 2015).

2. REVIEW AND THE LITERATURE

2.1 Thyroid Gland: Structure and Function

The thyroid gland is the largest endocrine gland, located in the anterior of the neck, above trachea and below the larynx (Figure. 1). In gross appearance, the thyroid gland is a bilobed, butterfly-shaped structure with two lateral lobes connected by a median tissue known as isthmus. The average weight of a normal adult thyroid gland is up to 20 g (Pankow et al. 1985). The thyroid gland is highly vascularized and has an adequate blood, lymph and nerve supply. The histology of the thyroid gland reveals that the structure is composed of two types of thyroid cells. One type is the follicular cells, which are simple epithelial cells and known as thyrocytes. These cells produce and secrete the thyroid hormones triiodothyronine (T3) and thyroxine (T4). In a layer, thyrocytes form follicles, which are filled with colloidal fluid containing thyroglobulin (Tg) protein and the stored thyroid hormones. The generation and secretion of T3 and T4 is governed by the thyroid-stimulating hormone (TSH). TSH binds to the TSH receptors and regulates the function of the thyroid gland. The second type of cells are the parafollicular C-cells which produce and secrete calcitonin, which regulates and maintains the blood calcium levels (Ritchie & Balasubramanian 2014). Although C-cells are situated between the follicles, they are scattered throughout the gland and mostly distributed medially. The thyroid gland is also known as the master gland, as the hormones secreted regulate most of the functions in the body, including growth and development (Stathatos 2012). In addition, there are four small parathyroid glands embedded on the posterior side of the thyroid gland (Figure. 1). The parathyroid glands produce and release parathyroid hormone (PTH) in response to low concentrations of calcium in the blood, which is sensed by the calcium sensing receptors (CSR). The stimulation of CSR terminates the release of PTH when the blood calcium homeostasis is achieved (Mihai 2014; Tovey et al. 2006; Potts 2005).

2.2 Thyroid disorders

The imbalance in the thyroid gland activity to produce or release thyroid hormones leads to thyroid diseases. Thyroid disorders include hyperthyroidism, hypothyroidism, inflammation of the thyroid or thyroiditis, thyroid nodules and thyroid cancers.

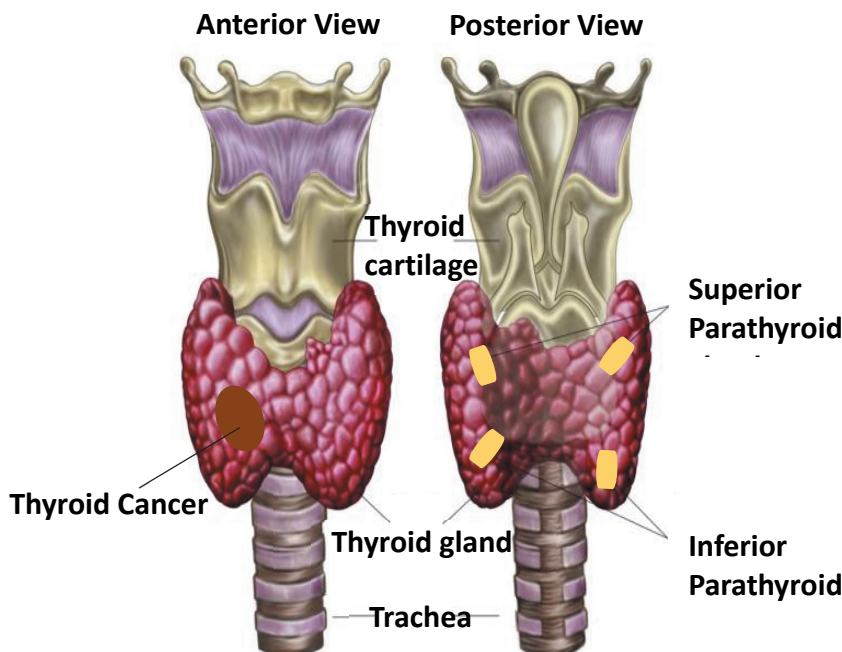


FIGURE 1. Structure and location of the thyroid gland. Taken and modified from website: <http://www.pathologyoutlines.com/topic/thyroidanatomy.html>. Accessed May 5th, 2017.

2.2.1 Hyperthyroidism

Hyperthyroidism is a condition in which the thyroid gland is hyperactivated and produces too much of the thyroid hormones T3 and T4. It causes an increased metabolic rate of the body which has been described to be associated with acute weight loss, heat intolerance, increased heartbeat, sweating, confusion, tremors and in some cases goiter and rarely atrial fibrillation. Graves' disease is a common cause of hyperthyroidism and is characterized by the production of an auto-antibody against the TSH receptor which causes increased production of thyroid hormones. The symptoms of Graves' disease depend on the age, onset, severity and duration of hyperthyroidism. In addition, the abnormalities in the production or secretion of the thyroid-stimulating hormone (TSH) from the pituitary gland or the thyroid-releasing hormone (TRH) from hypothalamus can result in hyperthyroidism (Smith & Hegedüs 2016).

2.2.2 Hypothyroidism

Hypothyroidism is a condition in which the thyroid gland is underactive and the production of the thyroid hormones T3 and T4 is low. The common symptoms include,

weight gain, lack of tolerance to cold, fatigue, and depression. Low production of the thyroid hormones greatly affects the normal functions of the body, including metabolism, growth and development. Subsequently, it affects the nervous (CNS), cardiovascular (CVS), gastrointestinal (GI) and reproductive systems. (Bello & Bakari 2012). The causes of hypothyroidism include iodine deficiency, the autoimmune disease Hashimoto's thyroiditis, damage to or a tumor in the hypothalamus or the anterior pituitary, and a partial or complete thyroidectomy. Goiter (enlargement of thyroid) is the abnormal growth of the thyroid gland, caused mainly by iodine deficiency or goitrogens, which results in malfunction of the thyroid gland. Thyroiditis is another thyroid disorder characterized by the inflammation of the thyroid. The most prevalent form is the Hashimoto thyroiditis in which patients develop goiter, the thyroid becomes underactive and the level of thyroid peroxidase (TPO) antibody is elevated (Sweeney et al. 2014).

2.2.3 Thyroid nodules

Ninety percent of thyroid nodules are small, non-palpable and benign which never transform into severe malignancy. However, the malignant tumors of the thyroid can be non-palpable or palpable. The common first method of diagnosis is fine needle aspiration (FNA) which may show if there are malignant cells present in the samples (Filetti et al. 2006; Popoveniuc & Jonklaas 2012). However, it is difficult to distinguish between a benign follicular thyroid adenoma (FTA) or malignant form of follicular thyroid cancer (FTC) based on FNA. Such patients first undergo partial thyroidectomy and then the correct diagnosis is made based on the histopathological examination of the biopsy. If FTC is confirmed there, then the patients undergo second surgery for complete thyroidectomy. Recent advancement of the use of high-frequency ultrasound devices has provided an excellent resolution and has enabled scientists to detect small size nodules up to 2-3mm through an ultrasound guided FNA (Guth et al. 2009). Such good advents have led to better screening and diagnosis of thyroid nodules.

2.2.4 Thyroid cancer

Thyroid cancer is the most common endocrine cancer and it is three times more common in females than in men. The incidences are rising throughout the world. The estimated annual number of new cases in the USA is 64,300 (American Cancer Society 2016). A similar rise in the incidences of thyroid cancer has been reported by the Finnish Cancer Registry over the last four decades, as shown in Figure 2.

The follicular thyroid cells, due to mutations or dedifferentiation, transform into either well differentiated follicular and papillary thyroid cancer cells or into dedifferentiated anaplastic thyroid cancer cells. However, the parafollicular or C cells transform into the medullary thyroid cancer cells (Dralle et al. 2015).

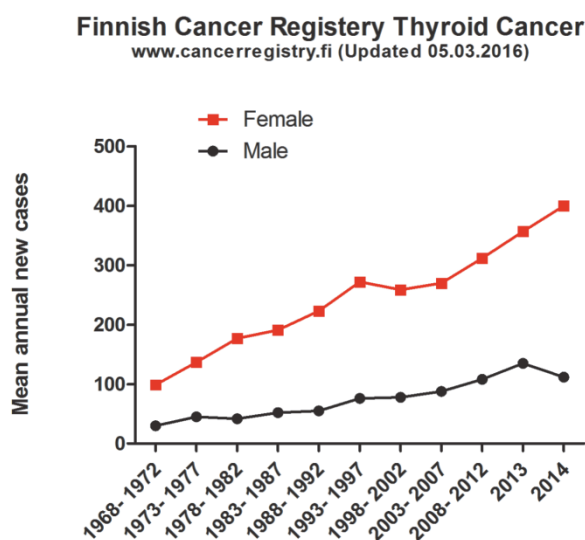


FIGURE 2. Incidences of thyroid cancer in Finland 1968-2014. www.cacerregistry.fi (Last Update, 05.03.2016).

2.2.4.1 Papillary thyroid carcinoma (PTC)

Papillary thyroid carcinoma (PTC) is the most common form (90% of all cases) of thyroid cancer. It is found to be more prevalent in young adults. In PTC, the tumor cells are diploid and well differentiated, grow slowly and respond well to treatment. The prognosis is often good and the survival for 20 years is 99% (Brito et al. 2014). The exposure to radiations and subsequent chromosomal rearrangements is the major cause of PTC. The genetic alterations in PTC include the BRAF^{V600E} (26-69%), RAS (0-21%), chromosomal rearrangements such as RET (13-25%), NTRK (5-13%) and ALK (12%) (Fagin & Wells 2016). These mutations, together with rearranged during transfection (RET) gene rearrangements, activate mitogen-activated protein kinase and extracellular regulated kinase ½ (MAPK/ERK1/2) pathways, which result in enhanced cell proliferation and migration. In addition, the BRAF^{V600E} mutation has been shown to be significantly associated with PTC recurrence, lymph node metastasis, and as an indicator of advanced stage of PTC (Tufano et al. 2012). Metastasis to bone and lungs is rare. However, the induction of metastases worsens the prognosis (LiVolsi 2011).

Furthermore, in 2013, the mutations in the telomerase reverse transcriptase (TERT) promotor were initially identified in thyroid cancer. These mutations occur frequently in PTC, FTC and ATC but not in MTC. These mutations have been established as a marker of aggressive thyroid cancer (X. Liu et al. 2013; T. Liu et al. 2013).

2.2.4.2 Follicular thyroid carcinoma (FTC)

Human follicular thyroid cancer (FTC) is the second most common, well differentiated and an aneuploid form of thyroid cancer. It has low prevalence (<10% of all cases of thyroid cancer) and usually has a good prognosis (Joensuu et al. 1986; Nikiforova et al. 2003). FTC are malignant tumors which are removed by partial or complete thyroidectomy. The RAS mutations (K-RAS, N-RAS and H-RAS) or rearrangements in the paired box gene 8/peroxisome proliferator-activated receptor γ (PAX-8-PPAR γ) manifest an aggressive phenotype resulting in enhanced tumor proliferation, and distant metastasis to bone and lungs is common (Martelli et al. 2002; Nikiforova et al. 2003).

Several studies have highlighted the importance of MicroRNAs (miRNAs), a class of short endogenous non-coding RNAs, in regulation of many cellular processes, including proliferation, apoptosis and differentiation. Several miRNAs have been shown to be upregulated and enhance proliferation and differentiation in differentiated forms of thyroid cancer, including papillary and follicular thyroid carcinomas (Weber et al. 2006; Rossing et al. 2012; Li et al. 2013).

2.2.4.3 Anaplastic thyroid cancer (ATC)

Anaplastic thyroid cancer accounts for 1-2% of all thyroid cancers. It is the most aggressive and highly invasive form of thyroid cancer. It is derived from the dedifferentiation of PTC or FTC cells. The prognosis is very poor and currently no effective treatment is available. ATC has a high invasive potential and metastasis to brain, lungs and bones is common. Due to this, the average life span is shortened to 3-5 months only after diagnosis. The mutations detected in anaplastic thyroid cancer cells are BRAF (rare 0-12%), RAS (20-60%), β -catenin (CTNNB1; 66%) and TP53 (67-88%). A combination therapy including surgery, chemotherapy, and radiation is in use for ATC patients and new drugs and combined strategies are urgently warranted to stop this deadly disease (Nagaiah et al. 2011; Smallridge et al. 2009; Carling & Udelsman 2014; Fagin & Wells 2016). In rare cases of ATC with BRAF-mutation (V600E), the BRAF inhibitor, Vemurafenib has a positive effect on prognosis. Furthermore, Everolimus, an inhibitor of the mammalian target of rapamycin (mTOR) has been suggested as a

possible therapeutic drug to treat metastatic ATC. However, prolonged use of this drug has been shown to induce an acquired resistance followed by a progressive disease (Rosove et al. 2013; Wagle et al. 2014).

2.2.4.4 Medullary thyroid cancer

Medullary thyroid carcinoma (MTC) accounts for 3 to 5% of all the thyroid cancers and usually occurs during the age of 40-60 years (Fagin & Wells 2016). In most of the cases (75%), MTC is sporadic and non-familial. The mutations include, typical RET point mutations, H-Ras, N-Ras and K-Ras (Moura et al. 2015). However, some rare mutations are also found in KIT, MET, fusion of RET and Alk (Ji et al. 2015; Grubbs et al. 2015).

MTC is the main component of the familial multiple endocrine neoplasia (MEN2) type 2 syndromes (MEN2A and MEN2B). MEN2A is the most prevalent (95% of the cases of MEN type 2) and has four subtypes, including classical MEN2A, MEN2A with Hirschsprung's disease, MEN2A with cutaneous lichen amyloidosis, and isolated familial medullary thyroid carcinoma (FMTC). Thus, At present MEN2A and MEN2B are the main types of MEN2 while FMTC is regarded as a variant of MEN2A The MEN2B syndrome is characterized by a distinct physical appearance including mucosal neuromas of the lips and tongue, marfanoid, medullated corneal nerves, and aerodigestive tract ganglioneuromatosis (Moline & Eng 2011; Fagin & Wells 2016). MEN2B is associated with the RET M918T mutation which displays a more aggressive phenotype than Ras mutations (Jasim et al. 2011). Genetic testing of RET is generally recommended for all MTC patients. In some cases of sporadic medullary carcinoma, MEN2B is expressed and genetic screening has a diagnostic value. The parafollicular C-cells from which the medullary thyroid carcinoma cells originate, do secrete calcitonin and carcinoembryonic antigen (CEA). These parameters are used as both screening markers for detection of MTC at early stages and as prognostic markers (Kloos et al. 2009; Carling & Udelsman 2014; Fagin & Wells 2016).

2.3 Sphingolipid Metabolism

Sphingolipids (SLs), a member of the huge lipid family, contain sphingoid bases (18 carbon aliphatic amino alcohols) as their characteristic structural units. Previously, SLs were known to provide only the structural integrity to the cell membranes. During the last decade, SLs have emerged as powerful bioactive signaling molecules, which have been established as regulators of cell physiology and pathology of the cells. Sphingolipid

metabolism is regulated and controlled by highly specific and conserved sphingolipid-generating and degrading enzymes. SLs are produced in two different ways: 1. De novo synthesis which is defined by the condensation of serine and palmitate, and 2. Sphingomyelin catabolism by specific enzymes. The sphingolipid metabolism has been summarized in Figure 3. Both pathways combine with the formation of ceramide, a central metabolite in the metabolism of SLs (Le Stunff et al. 2004; Hannun & Obeid 2008). Ceramide, sphingosine and sphingosine 1-phosphate are the most studied bioactive sphingolipids. The potential of interconversion makes these signaling moieties regulate a vast array of biological processes.

De novo synthesis begins with the condensation of serine and palmitoyl-CoA to 3-ketodihydrosphingosine. This reaction is catalyzed by serine palmitoyltransferase. Next, 3-ketodihydrosphingosine is reduced by 3-ketodihydrosphingosine reductase to dihydrosphingosine or sphinganine. Sphinganine is converted into dihydroceramide, catalyzed by ceramide synthase. Dihydroceramide is desaturated by dihydroceramide $\Delta 4$ -saturase to ceramide. Ceramide is used by the cell, or it is converted into sphingosine or sphingomyelin. Ceramide is converted to sphingosine by diacylation catalyzed by ceramidase. Sphingosine is phosphorylated on the C1-hydroxyl group by sphingosine kinases 1 and 2 (SK1 and SK2), and sphingosine 1-phosphate is produced. SK1 is abundantly present in the cytoplasm and is associated with the plasma membrane, endosomal vesicles and phagosomes. SK2 is predominantly present in the nucleus and less in the cytoplasm (Pitson et al. 2003; Igarashi et al. 2003; Shen et al. 2014). S1P is a highly bioactive lipid and regulates many processes in the cell. S1P is either converted back to sphingosine by sphingosine 1-phosphatase, or it is degraded by S1P lyase to hexadecenal and phosphorylethanolamine. Sphingomyelin is converted to ceramide by sphingomyelinase.

2.4 Sphingosine 1-phosphate and sphingosine 1-phosphate receptors

2.4.1 Sphingosine 1-phosphate (S1P)

S1P is a highly bioactive sphingolipid, composed of a polar head group (phosphate) and a long aliphatic sphingoid base backbone (Figure. 4). S1P regulates calcium release from the ER, cell trafficking, cytoskeleton rearrangements, survival, growth, migration and proliferation.

S1P is ubiquitously produced by all cells of the body. However, platelets, erythrocytes and immune cells have a huge amount of S1P accumulated. It is due to either the lack of

S1P lyase or due to the absence of the 1-phosphatase (Yatomi et al. 1995; Hänel et al. 2007; Ito et al. 2007). S1P is transported out of the cells through the ATP-binding cassette (ABC) and spinster 2 (Spins2) transporters present on the plasma membrane (Kim et al. 2009; Nishi et al. 2013; Nishi et al. 2014). In plasma, S1P is present in a high concentration (200 nM to 2 μ M) which is mostly bound to the apolipoproteins HDL and LDL but also to albumin. However, low concentrations of S1P has been observed in both lymph and interstitial fluid (Sattler & Levkau 2009; Rodríguez et al. 2009).

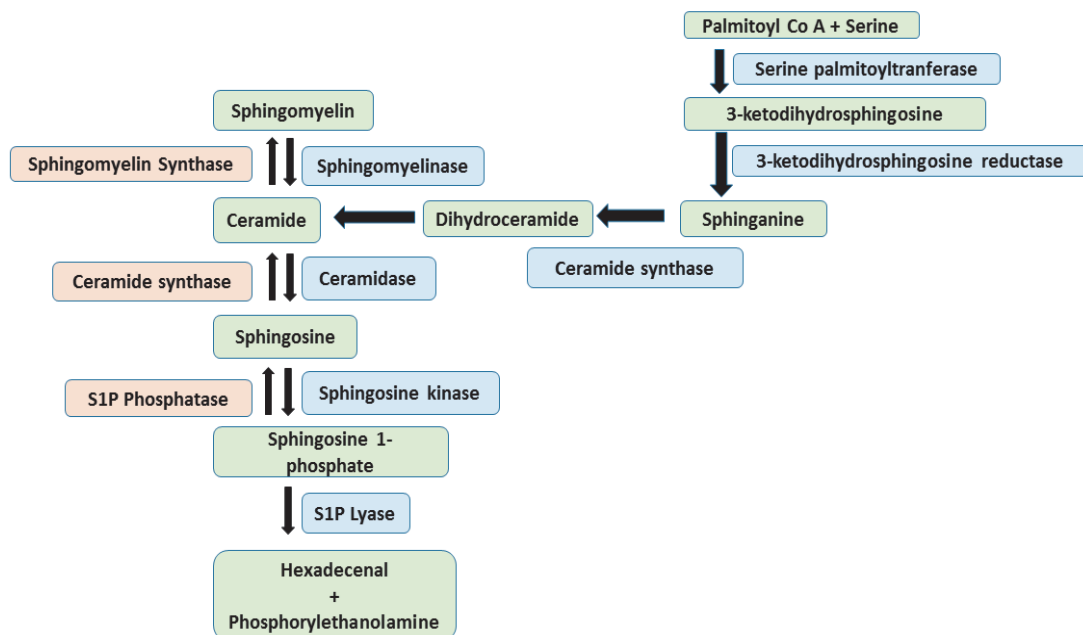


FIGURE 3. De novo biosynthesis of sphingolipids and S1P metabolism. Adapted and modified from Pyne & Pyne 2013; Cantalupo & Di Lorenzo 2016.

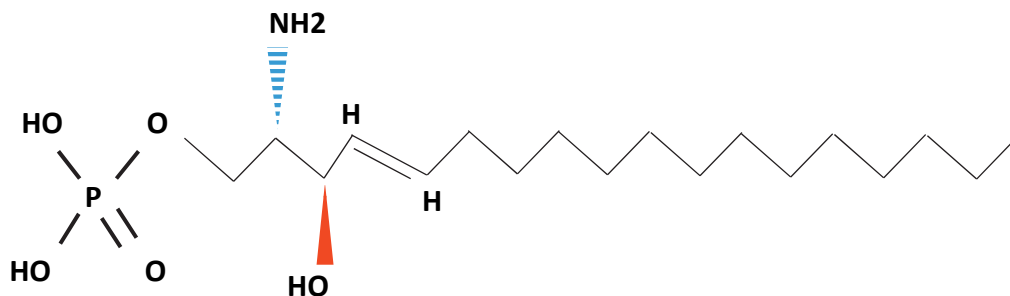


FIGURE 4. Chemical structure of sphingosine 1-phosphate (S1P). Molecular Formula: C₁₈H₃₈NO₅P.

2.4.2 S1P Receptors

Extracellular S1P binds with high affinity to five transmembrane GPCRs designated as S1P1-5. S1P acts both through an autocrine signaling loop and in a paracrine signaling manner, and activates downstream signaling pathways to regulate cellular responses (Pyne & Pyne 2013). S1P-receptors are a group of G-protein coupled receptors (GPCR) with seven transmembrane (TM) alpha helices connected through loop domains. Upon activation by extracellular S1P, GPCRs undergo a conformational change and activate the G protein complex ($G\alpha\beta\gamma$). GTP-bound to $G\alpha$ dissociates the G protein complex ($G\alpha\beta\gamma$) into $G\alpha$ and $G\beta\gamma$, which regulate downstream signaling pathways to obtain cellular responses. S1P receptor expression is highly specific for different cell types, but the cellular response depends exclusively upon which G protein subunit the receptor couples to and the subsequent activation of downstream pathways. S1P binds to receptors which couple to one of the four subunits $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ and regulate cellular responses by activation of distinct signaling pathways, including the Rho/ROCK and PI3K/Akt and Rac pathways, ERK1/2, adenylate cyclase (AC), Jun N-terminal kinase, phospholipase C (PLC) and intracellular calcium (Figure 5) (Lee et al. 2001; Im et al. 2004; Björklund et al. 2005; Lepley et al. 2005; Barber & Welch 2006; Vachal et al. 2006; Brinkmann 2007; Alvarez et al. 2007; Takabe et al. 2008; Sanz-Moreno et al. 2008; Y. M. Kim et al. 2011).

2.4.2.1 S1P1

S1P1 couples strongly and exclusively to the $G_{i/o}$ and is known for promoting tumorigenesis, tumor neovascularization, invasion and migration in many cancer cells. S1P1 mediates blood vessels formation both in the developing embryo and in cancer cells. The knockdown of S1P1, by either siRNA or inhibition using the pharmacological inhibitor FTY720, severely affects the development of mice embryo due to impaired vascularization, and vitiates tumor vascularization, respectively (Chae et al. 2004; LaMontagne et al. 2006). Similarly, in Lewis lung carcinoma cells and in mouse endothelial cells, knockdown of S1P1 attenuates tumor growth and migration (Chae et al. 2004). S1P1 promotes invasion and migration of many types of cancer cells by activation of the PI3K-Akt and the Rac pathways. For instance, in thyroid cancer ML-1 cells, S1P stimulates migration via S1P1 and downstream activation of PKC α , ERK1/2, the PI3K-Akt and Rac pathways (Balthasar et al. 2006; Bergelin et al. 2009). S1P1, via activating PI3K and Rac pathways, stimulates invasion and migration of a malignant

form of kidney tumor (Wilms tumor). In addition, S1P via S1P1 and downstream activation of Rac1, phosphorylates membrane-type matrix metalloproteinase 1 and promotes migration of malignant fibrosarcoma cells (Fisher et al. 2006; Li et al. 2009). S1P, by upregulation of S1P1 and a persistent activation of NF κ B and STAT3, regulates colon cancer by maintaining chronic colitis in mice (Liang et al. 2013). Thus, S1P1 is crucial and a driving force for tumor development, growth and malignancy (Pyne & Pyne 2013; Pyne et al. 2016). FTY720 (Fingolimod), which is an immunosuppressive and S1P1 receptor modulating drug mostly used for multiple sclerosis (MS), has been shown to effectively inhibit cell proliferation and migration of several cancer types, including thyroid cancer (Kalhori et al. 2016; White et al. 2016).

2.4.2.2 S1P2

S1P2 is ubiquitously expressed in all cell types and mediates a different array of cellular functions and pathologies. Extensive evidence suggests that S1P2 evokes an inhibitory and anti-tumorigenic effect on many cellular processes in normal and cancer cells. S1P2 couples to G $\alpha_{i/o}$, G $\alpha_{q/11}$ and G $\alpha_{12/13}$ but with a strong preference for G $\alpha_{12/13}$. S1P2 has been shown to regulate migration, proliferation, adhesion and angiogenesis. Thus, S1P2 regulates the function of the immune system, kidney, central nervous system, liver, vasculature and muscle cells (Adada et al. 2013). S1P inhibits proliferation of kidney tumor (Wilms) cells by activating S1P2 and downstream signaling pathways (Li et al. 2008). Stimulation of S1P2 causes stress fiber formation and disruption of cell junctions between endothelial cells. This effect is mediated by activation of the Rho-ROCK pathway and subsequent activation of the phosphatase PTEN, which inhibits the PI3K pathway (Sanchez et al. 2007). In rat pulmonary microvascular endothelial cells (PMECs), S1P via increased expression of S1P2 impaired wound healing and migration (Lu et al. 2012). In melanoma B16F10 cells, S1P was found to decrease migration via S1P2 and the Rho-ROCK pathway. Interestingly, Rac was inhibited upon Rho activation in these cells. In aggressive primary lung metastasis WiT49 cells, S1P evoked the expression of the anti-proliferative connective tissue growth factor (CTGF) via S1P2 (Li et al. 2008). The inhibitory effects of S1P2 on tumor growth and migration are highly cell specific and are associated with dominant expression of S1P2 and the downstream signaling pathways that it activates.

2.4.2.3 *S1P3*

S1P3 is expressed in all human cells; however, the most abundant expression of S1P3 has been detected in lung, heart and spleen. S1P3 couples to $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$ with a preference for $G\alpha_{i/o}$. This enables S1P3 to evoke similar responses as S1P1. S1P3 through $G\alpha_{i/o}$ activates the PI3K-Akt and Rac pathways to promote proliferation and migration and several other associated cellular processes. S1P3 also couples to $G\alpha_{q/11}$, thus activating phospholipase C (PLC β), which catalyses the formation of IP3 and DAG followed by activation of PKCs and calcium release from the ER to regulate many cellular processes (Neves et al. 2002). S1P3 regulates angiogenesis, proliferation and migration. S1P3 has been considered to be a potential inflammation marker to measure acute lung injuries (Sun et al. 2012). Together with S1P1, S1P3 induces inflammation of brain cells. Thus, pharmacological inhibitors of S1P3 could be potential candidates in the treatment of S1P3-associated diseases (Van Doorn et al. 2010). However, S1P3 has been shown to participate as an inhibitory modulator as well. S1P3 has been shown to modulate the migration and inflammatory potential by recruiting monocytes and differentiated macrophages to the site of inflammation (Keul et al. 2011). Furthermore, in vascular smooth muscle cells, S1P promotes vasoconstriction mediated by S1P3 and a subsequent increase in intracellular calcium (Murakami et al. 2010). In lung cancer cells, S1P3 together with EGFR regulates proliferation and migration (Hsu et al. 2012). In breast cancer cells, estrogen activates SK and the production of S1P. This endogenous S1P is transported out of the cells where it activates S1P3. The activated S1P3, in turn, transactivates EGFR and regulates proliferation and migration (Sukocheva et al. 2006). In astrocytes, S1P induces calcium signaling via the G_q -coupled receptors S1P2 and S1P3, which is followed by calcium influx through TRPC6 and activation of MAPK signaling and as a response, an increased secretion of the proinflammatory neuropeptide chemokine CXCL1 (Shirakawa et al. 2017a).

Taken together, coupling of S1P3 via G_q is essential for S1P-evoked PLC activation. S1P-induced PLC activation and the subsequent calcium mobilization mainly depend upon S1P3 expression in mouse embryonic fibroblast cells. Knockdown of S1P3 in these cells abolished this effect (Kon et al. 1999; Ishii et al. 2001; Ishii et al. 2002).

2.4.2.4 *S1P4*

The expression of S1P4 is highly tissue specific. Especially the hematopoietic and lymphoid organs express high concentrations of S1P4. S1P4 couples to $G_{12/13}$ more

strongly than to $G\alpha_{i/o}$. The response of S1P4 activation is cell specific. S1P causes cell rounding and activation of stress fibers via S1P4 coupled to $G_{12/13}$ and activation of the Rho pathway in Chinese hamster ovary (CHO) S1P4-overexpressing cells. S1P4 mediates the maturation of megakaryocytes in the bone marrow. However, in myoblasts, S1P4 induces apoptosis and muscle degeneration. S1P via S1P4 regulates T-lymphocyte proliferation, differentiation, migration and cytokine production (Gräler et al. 1998; Gräler et al. 2003; Golfier et al. 2010; Cencetti et al. 2013). S1P via S1P4 activates the ERK1/2 and HER2 pathways, which enhance the aggressive phenotype in estrogen receptor negative breast cancer MDA-MB-453 cells (Ohotski et al. 2012).

2.4.2.5 S1P5

There is comparatively little information about S1P5. In humans, S1P5 is expressed in the brain, leukocytes, lungs, spleen, placenta, aorta and fetal tissues. S1P5 couples to $G\alpha_{i/o}$ and $G\alpha_{12/13}$. S1P5 is expressed at low levels in the esophageal squamous cell carcinoma cell line Eca109. In Eca109 cells overexpressing S1P5, S1P inhibits migration and proliferation, while in serum starved prostate cancer PC-3 cells, S1P induces autophagic activity via an S1P5-activated pathway. (Chang et al. 2009; Hu et al. 2010). Furthermore, in glioblastoma cells, high expression of S1P5 has been shown to correlate with poor patient survival (Quint et al. 2014). Recently, S1P through activation of S1P5 has been reported to promote chromosome segregation and mitotic progression of Hela cancer cells (Andrieu et al. 2017).

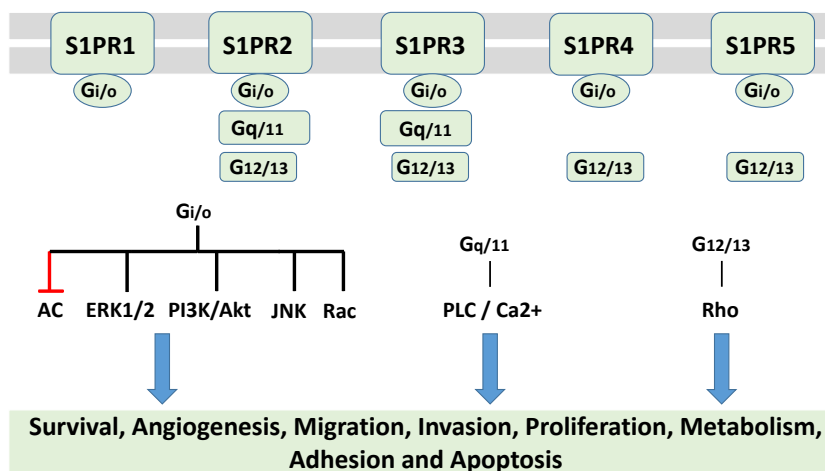


FIGURE 5. S1P-receptors and respective G-coupled downstream signaling molecules and effects. Modified from (Patmanathan et al. 2017).

2.5 HERG (Kv11.1) potassium channels in health and disease

The potassium channel family is the largest group of ion channels and has a broad and ubiquitous expression in all human cells, including the predominant expression in cardiac tissue. Based on the structure, potassium channels are divided into three main groups, a six-transmembrane domain structure (6TM), a two-transmembrane structure (2TM) and a four-transmembrane structure with two pore forming domains (4TM/2P). Voltage dependent potassium channels (Kv) belong to the 6TM family and have twelve subfamilies, Kv1-12. These channels play an important role in the repolarization of the action potential in both excitable and non-excitable cells. Kv channels, as the name indicates, open in response to the voltage change across bio-membranes. Some Kv channels are inactivated fast, while the others undergo a slow inactivation and are known as delayed rectifiers (Vandenberg et al. 2012; Arcangeli & Becchetti 2015). The human ether-a'-go-go related gene potassium channel (HERG, KCNH2, Kv11.1) belongs to the related eag-like family of potassium channels and the gene has been shown to encode an inward rectifying potassium channel. The term ether-a'-go-go came from a phenotype presented as leg shaking by a mutant *Drosophila melanogaster* in response to ether (Kaplan & Trout 1969; Warmke & Ganetzky 1994; Trudeau et al. 1995). In this thesis, HERG is used to denominate the human ether-a-go-go related gene for both mRNA and protein. These channels regulate many cellular functions to maintain normal physiology including heart rate, neuronal cell excitability, insulin secretion, cell proliferation and migration (Vandenberg et al. 2012). However, the genetic alterations or inhibition of HERG potassium channels have been shown to induce several pathophysiological conditions, including prolonged QT intervals, which increase the risk of fatal arrhythmia. Recently, it has been found that the loss of function mutation (p.T613A) in the outer pore region of the Kv 11.1 channel leads to the long QT syndrome (LQTS2), which is associated with severe arrhythmia and sudden death (Poulsen et al. 2015). HERG channels are overexpressed in several forms of cancers including acute myeloid leukemia (AML), colon and gastric tumors, endometrial cancer, brain tumors and adenocarcinomas. HERG regulates proliferation and migration in these cancer forms (Cherubini et al. 2000; Smith et al. 2002; Crociani et al. 2003; Lastraioli et al. 2004; Shao et al. 2008). The overexpression of HERG has been shown to correlate with poor prognosis as in e.g. aggressive glioblastoma multiforme (Masi et al. 2005). Previously in the Törnquist laboratory, we have reported that HERG channels are expressed in MDA-MB-435S melanoma cells and that the inhibition of HERG decreased the migration and

proliferation by obstructing the MAPK/c-fos pathway (Afrasiabi et al. 2010). Thus, HERG plays an important role in solid tumor progression and metastasis (Pardo & Stühmer 2014; Lastraioli et al. 2015).

2.6 Calcium signaling

Calcium is a ubiquitous intracellular second messenger and activates numerous cellular processes including fluid secretion, muscle contraction, exocytosis, gene transcription, fertilization, cell differentiation, proliferation and migration (Clapham 2007; Berridge et al. 2000; Pinto et al. 2015). The calcium signaling research field started in 1883, when Sydney Ringer, a British physiologist and pharmacologist, found that the contraction of frog heart muscle was enhanced by calcium in his saline solution. This was a purely accidental finding where Ringer used London tap water to prepare saline solution. The tap water was contaminated with calcium. This effect of calcium opened the door of the vast field of calcium signaling (Ringer 1883). The calcium signaling research has advanced remarkably during the past decades.

Calcium entry into the cells is activated either by depolarization of the plasma membrane and the activation of voltage-operated calcium channels (VOCs) such as in excitable cells, or in response to an agonist stimulus that through G coupled receptors activates membrane associated phospholipase C (PLC) and generates two important second messengers, inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). DAG has been shown to activate receptor-operated calcium entry (ROCE) by activation of TRPC channels, which enables calcium influx into the cells. The IP₃ diffuses into the cytoplasm and activates IP₃ receptors, resulting in the release of calcium from the ER stores. (Berridge et al. 2000; Venkatachalam et al. 2003; Montell et al. 2002; Venkatachalam & Montell 2007; Berridge 2016). The extracellular calcium concentration (up to 3 mM) is huge as compared to the low intracellular free calcium concentration at resting state. This big concentration gradient facilitates rapid calcium entry into the cells. The resting level of free calcium in the cytoplasm is maintained at low concentration, about 100 nM. Cells maintain this low level of calcium by strict regulation of calcium channels, pumps and calcium binding proteins. The calcium signals in cells are normally in the form of transients, as shown in Figs. 6 and 7. The abnormality or impairment of these calcium transients may induce severe diseases (Brini & Carafoli 2009; Stutzmann & Mattson 2011; Berridge 2016).

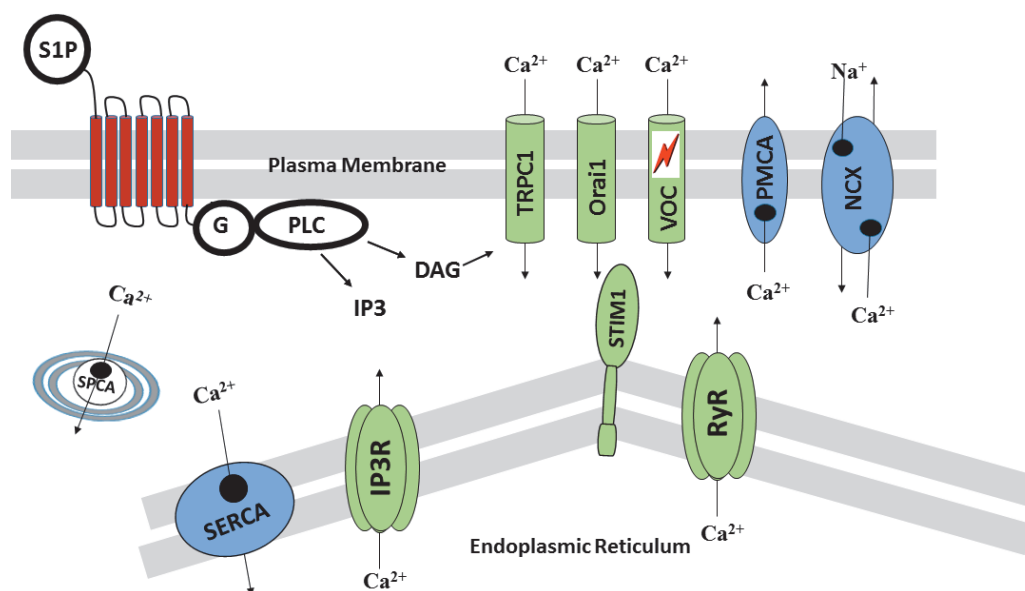


FIGURE 6. Mechanisms of calcium signaling. Upon activation of a GPCR receptor by a hormone or ligand, PLC is activated, which generates two second messengers: DAG and IP3. DAG is capable of activating TRPC channels in the plasma membrane and calcium influx is triggered. IP3 diffuses through the cytoplasm and binds to IP3 receptors on the ER membranes. This binding enables ER depletion and generates a transient calcium signal. The depletion of ER is sensed by STIM1 proteins which act as sensors. STIM1 makes a complex with Orai1 channels in the plasma membrane and induces store-operated calcium entry through Orai1. Voltage-operated calcium channels open in response to a depolarization of the plasma membrane in excitable neural and muscle cells. Calcium in the cytoplasm activates ryanodine receptors and calcium is released from the ER. To avoid a calcium flood in the cytoplasm, the pumps SERCA and PMCA, and the sodium calcium exchangers (NCX) are activated which export calcium out of the cell or into the ER. The secretory pathway calcium ATPase (SPCA) transports calcium ions into the Golgi apparatus.

The increase in the cytoplasmic calcium levels enables calcium-binding proteins such as EF-hand proteins, annexins and C2-domain proteins to become active and to regulate several cellular processes such as muscle contraction, exocytosis, metabolism, gene transcription, fertilization, proliferation and hypertrophy. To avoid too much of calcium in the cytoplasm, the levels of calcium in the cytoplasm are maintained for a time range of micro-second to hours, depending upon the nature of the cell type and the specific function that is being carried out. Persistently elevated calcium levels in the cytoplasm can lead to apoptosis and cell death. Moreover, irregular, high, or low amplitude calcium signals have been associated with diseases.

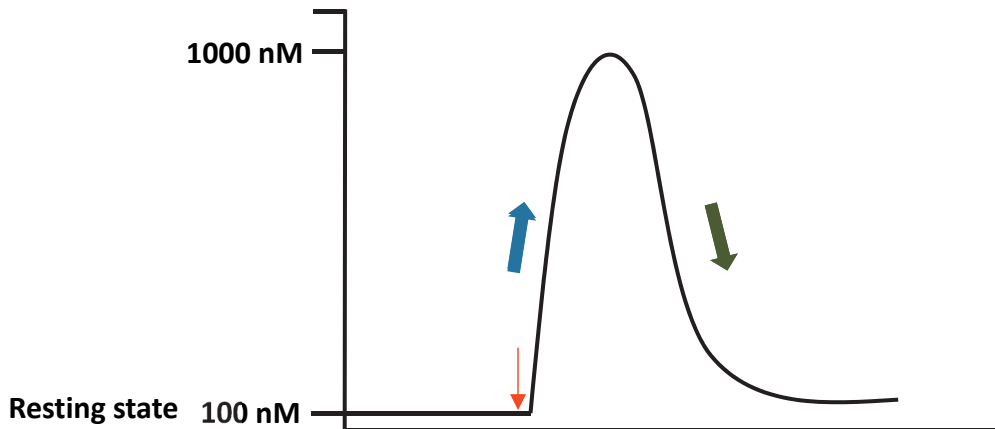



FIGURE 7. A calcium signal is depicted. The red arrow depicts the stimulus or agonist, which activates either calcium entry or release or both. The blue arrow indicates the rapid increase of calcium in the cytoplasm up to tenfold. The green arrow represents a rapid inactivation of calcium channels and an activation of calcium pumps present on the plasma membrane or on the ER to avoid the cell being flooded by calcium. These mechanisms bring the free calcium level back to the normal, resting level.

2.7 Transient Receptor Potential (TRP) channels

In the fly *Drosophila melanogaster*, a mutant which caused temporary blindness in bright light stimulus was detected and later it was found that this mutation encodes a cation TRP channel (Cosens & Manning 1969; Minke et al. 1975; Montell & Rubin 1989). The transient receptor potential (TRP) channel-superfamily is the largest family of cation channels and is comprised of 28 channels in mice and 27 in humans, as shown in Table 1.

TABLE 1. Expression of TRP channels in worms, flies, mice and human.



	Worm	Fly	Mouse	Human
TRPC	3	3	7	6
TRPV	5	2	6	6
TRPM	4	1	8	8
TRPA	2	4	1	1
TRPML	1	1	3	3
TRPP	1	1	3	3
TRPN	1	1	0	0
Total	17	13	28	27

The expression and function of TRP channels is diverse throughout the animal kingdom including worms, flies, mice and human. There are seven subfamilies of TRP channels based on amino acid sequence homology, and include TRPC, TRPV, TRPM, TRPA, TRPP, TRPML and TRPN as shown in Figure 8.

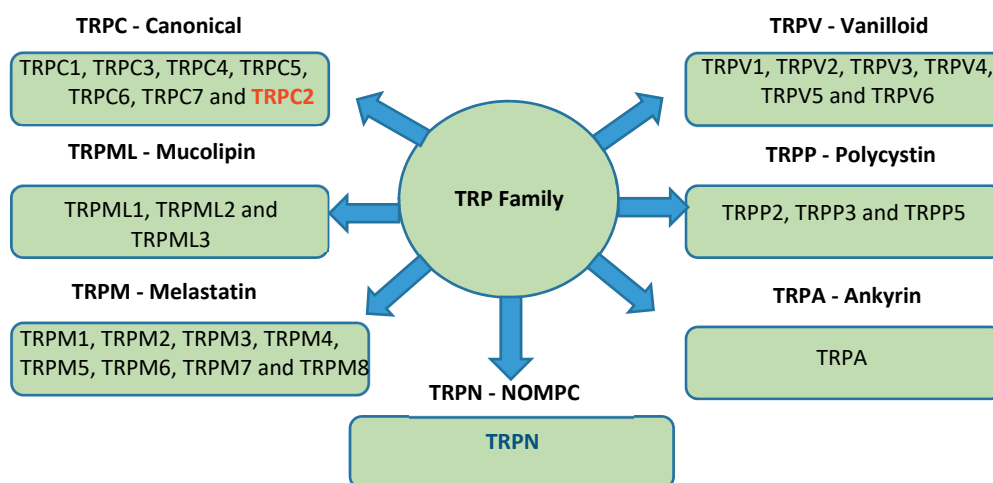


FIGURE 8. The human phylogenetic tree of the TRP channel superfamily. TRPC (canonical), TRPV (Vanilloid), TRPML (Mucolipin), TRPP (Polycystin), TRPM (Melastatin), TRPA (Ankyrin) and TRPN (NOMPC). TRPC2 (red) is a pseudogene in human. TRPN (blue) is expressed in fish. Modified from (Nilius & Owsianik 2011).

TRP channels are selective to cations and some subtypes are highly selective for calcium and magnesium (Mg^{2+}). The channels are ubiquitously expressed in human tissues.

Many TRP channels have been indicated to participate in a multitude of physiological processes including perception of heat, touch, pain, odor and smell, cellular regulation of osmolarity, fluid secretion, inflammation, cell adhesion, proliferation, cell differentiation, migration and apoptosis (Clapham 2003; Clapham 2007; Julius 2013; Montell 2001; Minke & Cook 2002; Minke 2010; Nilius & Mahieu 2006; Benemei et al. 2015; Nilius & Flockerzi 2014; Kaneko & Szallasi 2014; Gees, Owsianik & Voets 2012). In addition to the physiological importance, TRP channels have been found to regulate many diseases including cancer (Nilius 2007; Prevarskaya et al. 2007; Van Haute et al. 2010; Lehen'Kyj & Prevarskaya 2011; Ouadid-Ahidouch et al. 2013).

This thesis work is focused to investigate the identity of TRPC channels in human normal thyroid tissue and in thyroid cancer tissue and cell lines.

2.7.1 TRPC Channels

The transient receptor potential canonical (TRPC) subfamily consists of seven members (TRPC1-7). In humans, all isoforms are expressed except TRPC2, which is a pseudogene. TRPCs are non-selective cation channels, with a preference for calcium over sodium and potassium ions. The human TRPC channels are more closely related to the *Drosophila* TRP channels. The structure of the TRPC channels consists of six transmembrane segments (S1-S6) connected through loops, and the amino (N) and the carboxyl (C) terminals are located inside the cytoplasm. The length and amino acid sequence of the C or N terminals varies. The loop region between segments 5 and 6 makes the pore, which conducts ions, as shown in Figure 9. The N-terminus has a coiled-coil domain and four ankyrin domains, which facilitate protein interactions and have been shown to be involved in the regulation of TRPC channel function by tetramerization of TRPC subunits (Lepage et al. 2006). The C-terminus has a TRP domain, which is the site for other TRP channel isoforms to bind and form channel complexes, a coiled-coil domain and a calmodulin and IP3R binding site, which regulate the activation and inhibition of the channel (Brinbaumer 2009). TRPCs regulate several calcium-dependent cellular processes. TRPC1 can co-assemble with all other TRPC isoforms (TRPC3-7). The TRPC1/TRPC3 complex enhances store-operated calcium entry, and regulates the differentiation of e.g. H19-7 hippocampal neuronal cells (Wu et al. 2004). TRPC channels participate in both receptor-operated calcium entry and store-operated calcium entry together with Orai1 and STIM1 proteins.

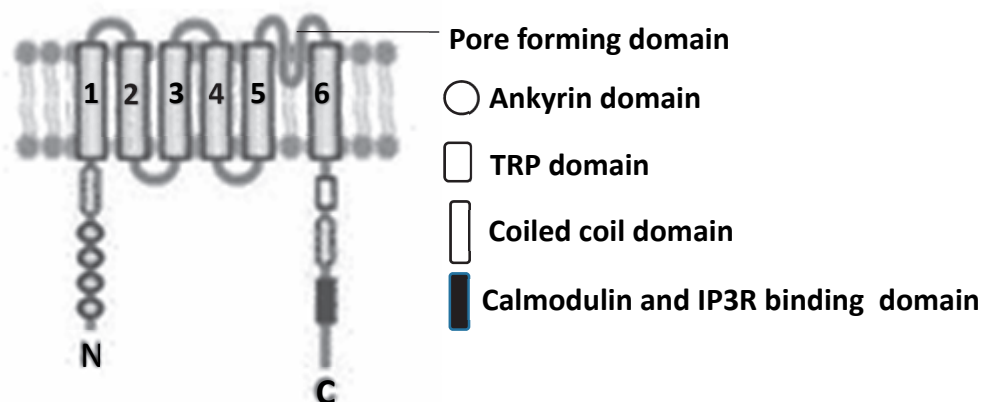


FIGURE 9. TRPC channel transmembrane structure and the domain organization of the subunits.

2.7.1.1 TRPC1

TRPC1 channels are ubiquitously expressed in human tissues and participate in carrying out many cellular processes including cell migration and proliferation (Ricchio et al. 2002; Louis et al. 2008; Abramowitz & Birnbaumer 2009; Kuang et al. 2012). TRPC1 is the first mammalian TRPC channel identified and cloned (Wes et al. 1995). TRPC1 has been established as a potent molecular component of SOCE in several distinct cell types (Parekh & Putney 2005; Beech 2005; Ambudkar 2007; Venkatachalam & Montell 2007). In addition, TRPC1 has been shown to function as a receptor-operated calcium entry channel in several cell lines (Storch et al. 2012; Nesin & Tsiokas 2014). TRPC1 channels are essential for the polarity and direction of migrating cells both *in vitro* and *in vivo*. In aggressive glioma cells, TRPC1 regulates EGF-evoked migration. In addition, TRPC1 has been shown to localize to lipid rafts at the leading edge of migrating cells. TRPC1 mediated calcium entry has been shown to activate the MAPK/ERK1/2 and PI3K/Akt signaling pathways, calpains, HIF1 α and MMPs. Thus, TRPC1 has emerged as an important player involved both in normal and cancer cell function (Bomben & Sontheimer 2008; Rao et al. 2006). TRPC1 can make complexes with all other TRPC channels. In HSY cells, TRPC1 forms a complex with TRPC3. In HEK293 cells, TRPC1 binds with TRPC3 and TRPC7 to form a heteromeric complex. In mesangial cells, TRPC1 forms a complex with TRPC4 and in neuronal cells TRPC1 has been shown in complex with TRPC5 (Liu et al. 2005; Rao et al. 2006; Bomben & Sontheimer 2008; Louis et al. 2008; Dhennin-Duthille et al. 2011). TRPC1 channels also interact with the calcium-signaling proteins G_{q/11}, PLC, CaM, IP3R, PMCA, SERCA and STIM1 to

regulate cellular processes. STIM1 and the TRPC1-TRPC4 complex are essential for store refilling and differentiation in myotubes (Antigny et al. 2017).

2.7.1.2 TRPC2

TRPC2 is a pseudogene and does not form a functional channel in humans (Wes et al. 1995; Wissenbach et al. 1998). Little is known about this channel's physiology and pathology. However, in other mammals TRPC2 forms a functional channel in distinct tissues like the vomeronasal organ (VNO), testis, spleen and liver (Wissenbach et al. 1998; Vannier et al. 1999). TRPC2 has been shown to interact with Homer1, CaM, $G_{q/11}$ and IP3R (Yuan et al. 2003; Lockwich et al. 2000). In the Törnquist laboratory, we detected TRPC2 for the first time in rat thyroid FRTL-5 cells. The knockdown of TRPC2 or expression of a dominant negative form isoform of TRPC2 in these cells resulted in a significant decrease in proliferation, migration, adhesion and invasion (Sukumaran et al. 2013). In addition, TRPC2 has been reported to mediate both receptor-operated calcium entry (ROCE) and store-operated calcium entry (SOCE) in FRTL-5 cells (Törnquist et al. 2014).

2.7.1.3 TRPC3

TRPC3 channels are expressed in the human brain, kidney, skeletal muscle, mammary, ovary and cardiovascular tissues (Abramowitz & Birnbaumer 2009). TRPC3 interacts and forms heterodimers with TRPC1, 4, 5, 6, and -7. Despite a considerable homology of the amino acid sequence between TRPC3, TRPC6 and TRPC7, these channels regulate many different functions in humans (Putney 2005; Owsianik et al. 2006). TRPC3 also interacts with the Orai1 calcium channel (Liao et al. 2009; Woodard et al. 2010). TRPC3, either in complex with TRPC1, or in complex with both TRPC1 and TRPC7, forms a SOCE channel. TRPC3 interacts with several signaling proteins in response to receptor-evoked calcium mobilization, including $PLC\beta$, $G_{q/11}$ and IP3R. TRPC3 has been reported to be directly activated by DAG (Trebak et al. 2003).

2.7.1.4 TRPC4

TRPC4 shares a 65% homology in the amino acid sequence with TRPC5 (Parekh & Putney 2005; Venkatachalam & Montell 2007). Mostly, TRPC4 channels form SOCE channels but in some cell types they form store-independent channels as well. It can make heteromeric channels with TRPC6. TRPC4 can directly activate STIM1. TRPC4 has been studied extensively in regard to endothelial cell function and has been shown to regulate proliferation of these cells (Abramowitz & Birnbaumer 2009).

2.7.1.5 TRPC5

TRPC5 is highly expressed in neuronal cells and regulates their functions. In human embryonic kidney (HEK) cells, TRPC5 is a non-selective channel and functions as a receptor-operated channel but not as a store-operated channel (Schaefer et al. 2000). Recently, it has been shown that TRPC5 is activated in response to mechanical stress, and this increase in the activity of the channel is dependent on actin filaments (Shen et al. 2015). TRPC5-TRPC1 complexes are found in neurons, vascular endothelial cells and smooth muscle cells. In vascular smooth muscle (VSM) cells, TRPC5 has been suggested to act as SOCE channel by forming complexes with TRPC1, TRPC6 and TRPC7 (Earley & Brayden 2015).

2.7.1.6 TRPC6

TRPC6 is expressed in pulmonary and vascular smooth muscle cells. These channels are directly activated by DAG and do regulate receptor-operated calcium entry (Dietrich et al. 2005). However, activation of TRPC6 due to calcium release from the ER is mediated by Orai1 and TRPC4 (Liao et al. 2007). TRPC6 has been shown to make a complex with TRPC3 in neuronal cells and prostate cancer epithelial cells. In astrocytes, on activation, TRPC6 activates MAPK signaling which results in an increased secretion of neuroprotective CXCL. Furthermore, TRPC6 is important in TGF β 1 signaling in vascular smooth muscles. TGF β 1 induces stress fiber formation in these cells via upregulation of TRPC6 (Shirakawa et al. 2017a). In human prostate cancer epithelial (hPCE) cells, active TRPC6 and NFATs promote proliferation via alpha 1-AR signaling (Thebault et al. 2006).

2.7.1.7 TRPC7

In humans, TRPC7 is widely expressed in many tissues including the brain, skin, cartilage, pituitary gland, intestine, kidney and prostate (Abramowitz & Birnbaumer 2009). TRPC7, a non-selective cation channel is the seventh identified member of mammalian TRPC family. It was isolated through molecular cloning of the mouse fetal brain and caudate nucleus cDNA libraries (Nagamine et al. 1998; Okada et al. 1999). The role of TRPC7 in regulation of normal cell physiology and pathology is still indefinable. TRPC7 is activated by G_q-coupled protein receptors and the PLC pathway, and is directly activated by DAG. In some cell types, TRPC7s are constitutively active proteins and may function as SOCE channels by forming a TRPC1-TRPC3-TRPC7 complex. However, in HEK-293 cells, a TRPC3 and TRPC7 complex is activated by DAG and functions as

ROCE channels (Zagranichnaya et al. 2005). Furthermore, cGMP-dependent protein kinase 1 α , CaM, IP3R and PIP2 have all been reported to regulate the function of TRPC7 (Yuasa et al. 2011). In addition, activation of TRPC7 potently induces myocardial apoptosis (Sato et al. 2007).

2.8 Malignant Tumor and Metastasis

Cancer or a malignant tumor is associated with an uncontrolled proliferation, massive growth and invasion of the cells or tissue. How can cancer cells acquire such a phenotype? It is a multistep process, which starts with mutations and alterations in the genes which is followed by the activation of early-embryonic stage transcription factors for cell growth and development. These specific transcription factors upregulate the expression of several genes and proteins which enable the cell to change its shape by cytoskeleton rearrangements, activate the migratory pathways to acquire motility, and the production and secretion of extracellular matrix (ECM) degrading enzymes which make cancer cells more invasive. The cytoskeleton rearrangements include the formation of lamellipodia and filopodia by the activation of the small GTPases CDC42 and Rac1 and associated signaling pathways, which govern actin polymerization and concentration of focal adhesions at the leading edge of the invading cell. The RhoA GTPase is found at the rare or trailing edge, where it controls the formation of stress fibers and focal adhesions. The retraction of the rare edge is achieved by the disassembly of focal adhesions through the action of ECM degrading proteolytic enzymes including matrix metalloproteinases (MMPs). The cell moves by contraction of the cell body through Rho and ROCK signaling. These contractions of actin-myosin complexes result in the tail detachment and movement of the cell towards the leading edge (Lauffenburger & Horwitz 1996; Parri & Chiarugi 2010; McAllister & Weinberg 2014; Ridley 2015; Sadok & Marshall 2014; Zegers & Friedl 2014).

Thus, cancer cells acquire the ability to leave the primary tumor and invade locally adjacent tissues, or invade the blood stream. This is known as intravasation. The cancer cells are transported through blood and lymph to distant sites, where they escape from the blood stream and enter the tissue. This is known as extravasation. Cancer cells have a remarkable potential to adapt to the conditions of the new tissue environment, proliferate and grow to form metastases. This whole sequence of steps is known as the invasion-metastasis cascade. The cancer cells in the tumor change from an epithelial, differentiated state to a mesenchymal state, also known as the epithelial-mesenchymal transition (EMT). EMT is characterized by the loss of epithelial morphology,

cytoskeleton rearrangements, lower expression of epithelial genes and proteins including E-cadherin, the activation of mesenchymal genes and associated proteins including vimentin and N-cadherin, and acquisition of a mesenchymal shape. However, in the metastases, due to lack of the signal proteins, the expression of the early-stage embryonic transcription factors is ceased and cancer cells revert from the mesenchymal to the epithelial state, also termed as reversal of the EMT or mesenchymal-epithelial transition (MET). Such cells may remain dormant for long periods of time, but have a potential to acquire again the aggressive malignant phenotype (Tiwari et al. 2012; Grant & Kyprianou 2013; Heerboth et al. 2015; McAllister & Weinberg 2014; Sevenich & Joyce 2014).

2.9 Extracellular Matrix (ECM) Degrading Enzymes

The extracellular matrix (ECM) is the major component of the cellular microenvironment and is composed of many structural proteins, which are well organized and provide strength to the tissue structure and regulate the maintenance of the homeostasis. The structure of the ECM is highly dynamic and the components continuously undergo synthesis, modification or degradation. The dynamic range in the structure is the back-bone of the diverse functions performed by the cells in specific tissue. The ECM regulates many cellular processes including proliferation, adhesion, invasion and migration (Hynes 2009, Harburger and Claderwood 2009). The ECM can be divided into two groups, interstitial connective tissue and the basement membrane. The interstitial connective tissue matrix, which is mainly composed of collagen I and fibronectin, surrounds the cells and provides support to the tissue. The other part of the ECM is the basement membrane, which is more compact and composed of mainly collagen IV, laminins, and other proteins secreted by the epithelial cells. The basement membrane is a specialized form of the ECM that not only separates the epithelium from the stroma, but also regulates the interactions between the ECM proteins and the cell surface receptors to carry out a multitude of cellular and tissue functions (Mouw et al. 2014). However, an abnormality or impairment in the ECM composition, structure or consistency leads to pathological conditions such as fibrosis and invasive cancer (Bonnans et al. 2014). Malignant cancer cells produce and secrete several ECM degrading enzymes which hydrolyze the basement membrane and facilitate cell invasion. Matrix metalloproteinases (MMPs) are the main and well-documented enzymes active in ECM remodeling and degradation (Cawston & Young 2010).

2.10 Matrix metalloproteinases (MMPs)

In 1962, Jerome Gross and Charles M. Lapiere for the first time described the remodeling and degradation of collagen by collagenolytic enzymes during tadpole tail metamorphosis (Gross & Lapiere 1962). MMPs are calcium-dependent zinc-containing endopeptidases and belong to the large family of ECM degrading proteinases. To date, there are 23 members identified which have been divided into subgroups depending upon their structure and localization in the cell. For detailed structure and their specific substrates, see Table 2. The MMPs share some common functional domains including a signal peptide (SP; except in MMP-23A and -23B), a pro-peptide (PP), a catalytic domain containing zinc (CD (Zn)) and a hemopexin domain (HPX; except in MMP-7, 26, 23A and 23B). These domains make the basic structure of the MMPs, as shown in Figure 10. However, MMP-7 and -26 have only the minimal domain, including the signal peptide, the pro-peptide and the catalytic domain containing zinc. In addition, some MMPs may have furin, fibronectin and glycosylphosphatidylinositol (GPI) anchors and trans-membrane domains (TM), which enable specific activity of MMPs on different substrates. The structures of MMP-23A and MMP23B are unique, as they contain an amino terminal signal anchor (SA), a trans-membrane domain (TM), a cysteine rich domain (Cys) and an immunoglobulin-like domain (Ig), which allow their action on gelatin as substrate (Egeblad & Werb 2002; Kessenbrock et al. 2010; Lu et al. 2011; Bonnans et al. 2014). Altogether, these MMPs are sufficient to break down all the ECM proteins. MMPs are secreted in an inactive zymogen form which are converted into active MMPs. Pro-MMPs are cleaved by serine proteases, active MMPs or via oxidation of thiol groups of cysteine residues in the pro-peptide domain. The oxidation is made by reactive oxygen species (ROS), which are produced by neutrophils and macrophages in response to the inflammation at the tumor site (Weiss et al. 1985; Lu et al. 2011). MMPs are highly specific and cleave only the respective substrates. In normal cells, the secretion of MMPs is low as compared to a substantial secretion in inflammation or cancer. MMP3 and MMP10 have been reported to cleave and activate pro-MMP1, 8 and 13. Similarly, MMP14, 16, 24 and 25 can activate pro-MMP2. In addition, MMP12 has been shown to activate pro MMP13 (Page-McCaw et al. 2007; Kessenbrock et al. 2010).

Most of the secreted MMPs are in soluble form except for the membrane-type MMPs (MT-MMP14, 12, 23 and 24) and the glycosylphosphatidylinositol (GPI) linked MMP17 and 25. Due to the wide spectrum of action on numerous substrate targets, MMPs have an ability

to regulate a wide range of physiological and pathological functions including tissue remodeling, inflammation, angiogenesis, invasion and migration (Kessenbrock et al. 2010).



FIGURE 10. Basic structure of metalloproteinases (MMPs) (Bonnans et al. 2014).

2.10.1 Regulation of MMPs.

The understanding of MMPs' function in regulation of cancer metastasis has advanced considerably during the past decades. MMPs have been indicated as the principal mediators that regulate the alterations in the microenvironment to facilitate tumor growth and migration (Egeblad & Werb 2002; Page-McCaw et al. 2007; Kessenbrock et al. 2010; Shuman Moss et al. 2012). The activity of MMPs is dynamic and strictly regulated. The functions of active MMPs include the regulation of transcription, the activation of zymogen and their intra/extracellular localization and compartmentalization. The regulation of the MMP genes at the transcriptional level is regulated by cytokines and growth factors. However, the posttranscriptional gene regulation of MMPs is controlled by small non-coding RNA molecules, microRNAs. microRNAs bind to the post-transcriptional transcripts of MMPs and either suppress their activity or block and degrade them, thus, influencing the translation and expression of MMP genes (Li & Li 2013; Esquela-Kerscher & Slack 2006). The ECM degradation is maintained under strict control to avoid excessive and harmful tissue degradation in normal cells. For this purpose, cells express and secrete endogenous inhibitors to inactivate the secreted active MMPs. These highly specific inhibitors are known as the tissue inhibitor of metalloproteinases (TIMP). There are four members of the TIMP inhibitor family, TIMP1-TIMP4 (Bonnans et al. 2014). The binding ratio of TIMP to MMP determines the degradation pattern of the ECM. However, in case of malignant cancer cells, where cells secrete massive amounts of MMPs, the effect of TIMPs is overthrown. TIMP1 expression has been reported to decrease the proliferation and migration of colon cancer cells (Song et al. 2016). Similarly, TIMP2, which regulates the activity of MMP2, was transfected in rat mammary adenocarcinoma ENU1564 cells, decreased the proliferation and metastasis of these cells (Mendes et al. 2007). Overexpression of TIMP3 in human osteosarcoma Saos2 and MG63 cells, inhibited migration and invasion. However, knockdown of TIMP3 enhanced migration of these cells (Han et al. 2016). In addition, α_2 -macroglobulin, a glycoprotein present in the

blood, is a potent inhibitor of MMPs. α_2 -Macroglobulin not only inhibits MMPs but also binds to several important proteins and growth factors to regulate their activity (Rehman et al. 2013). The emerging knowledge about the regulation and functions of these proteolytic enzymes has opened a new gateway to design potent pharmacological inhibitors and to adopt useful strategies to cure aggressive cancers.

2.10.2 MMP2 and MMP9 in cancer

MMP2 (Gelatinase A) and MMP9 (Gelatinase B) are the most profound MMPs indicated in cancer cell migration. These are secreted into the extracellular space and have an ability to break down gelatin. However, the main substrate of these MMPs is collagen IV, which is the major component of the basement membrane in the extracellular matrix. MMP2 and MMP9 regulate several important cellular processes such as cell proliferation, migration and invasion, angiogenesis and inflammation by degradation of several important regulatory proteins and the ECM. The structural domain hemopexin C of MMP2 and MMP9 interacts with cell surface anchored proteins such as integrins and regulate migration. The role of S1P in regulation of MMP2 and MMP9 is well documented (Bergers et al. 2000; Stamenkovic 2000; Song et al. 2015; Mira et al. 2004; McCawley & Matrisian 2000; Khokha et al. 2013; Manicone & McGuire 2008).

2.10.3 Calpains

Calpains (CAPNs) were discovered as calcium-activated neutral proteases extracted from rat brain (Guroff 1964). The primary structure of these calcium proteases revealed that they are made up of four specific domains. Interestingly, domain 2 and domain 4 were found to be similar to the papain-like thiol protease and the calmodulin-like calcium binding protein (Ohno et al. 1984). Calpains are non-lysosomal, ubiquitously expressed proteases, and are among the few proteins in the cells, which are directly activated by calcium. In humans, 15 calpain genes are expressed which yield into more than 50 transcripts (some of these genes generate more than one transcript which encode different variants). Despite that so many calpains have been identified, the conventional calpain-1 (also known as μ -calpain) and calpain-2 (m-calpain) are the most extensively studied isoforms. Calpains are activated in response to high intracellular calcium concentrations and regulate different cellular functions including cell migration and proliferation, differentiation, cytoskeletal remodeling and apoptosis (Ono et al. 2016). On activation, calpain-1 and calpain-2 partially cleave a broad spectrum of substrates including proteins and several transcription factors to modulate cellular functions. Calpastatins (CASTs), the

highly specific and potent inhibitors of calpains, are endogenously produced by the cells that enable a limited proteolysis of target proteins (Leloup & Wells 2011).

TABLE 2. Structure of mammalian matrix metalloproteinases (MMPs). Modified from; (Lu et al. 2011; Bonnans et al. 2014).

MMP designation	Common names	ECM Substrates
Basic domains structure (SP + PP + CD (Zn) + HPX)		
MMP 1	Collagenase 1, interstitial collagenase, fibroblast collagenase, tissue collagenase	Collagens, entactins, PGs, ovostatin, MMP2, MMP9, pro-MMP9
MMP 3	Stromelysin-1, transin-1, proteoglycanase, procollagenase-activating protein	Collagens, gelatin, aggrecan, laminin, elastin, casein, osteonectin, fibronectin, entactins, Plasminogen, ovostatin pro-MMP9
MMP 8	Collagenase-2, neutrophil collagenase	Collagens, fibronectin, PGs
MMP 10	Stromelysin-2, transin-2	Collagens, gelatin, elastin, casein, fibronectin
MMP 12	Metalloelastase, macrophage elastase	Collagen IV, elastin, gelatin, casein, fibronectin, vitronectin, laminin, entactin, fibrinogen
MMP 13	Collagenase-3	Collagens, tenascin, plasminogen, aggrecan, fibronectin, osteonectin, MMP9
MMP 19	RASI-1	Collagen I and IV, gelatin, fibronectin, laminin
MMP 20	Enamelysin	Amelogenin
Minimal domains structure (SP + PP + CD(Zn))		
MMP 7	Matrilysin, matrin, small uterine metalloprotenase	Collagen IV, elastin, gelatin, casein, fibronectin, laminin, transferrin
MMP 26	Endometase, matrilysin-2	Collagen IV, fibrinogen, fibronectin, gelatin, pro-MMP9
Furin domains (SP + PP + Fur + CD (Zn) + HPX)		
MMP 11	Stromelysin-3	Collagens, fibronectin, PGs, laminin, elastin, casein
MMP 21	Homologue of Xenopus XMMP	Not Detected
MMP 28	Epilysin	Casein
Fibronectin domains (SP + PP + CD (Zn) + FN + HPX)		
MMP 2	Gelatinase A, 72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase	Collagens (IV,V,VII,X), gelatin, elastin, fibronectin
MMP 9	Gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase	Collagens (IV,V,VII,X), gelatin, elastin, fibronectin
Membrane-bound MMP anchored by GPI structure (SP + PP + CD (Zn) + HPX + GPI)		
MMP 17	MT-4 MMP, MT-MMP4	Gelatin, fibrinogen, pro-MMP2
MMP 25	MT-6 MMP, MT-MMP6, leukolysin	Collagen IV, Gelatin, fibronectin, pro-MMP2
Trans-membrane bound domains structure (SP + PP + CD (Zn) + HPX + TM + CT)		
MMP 14	MT1-MMP, MT-MMP1	Collagens, gelatin, fibronectin, laminin, vibronectin, entactin, pro-MMP2
MMP 15	MT2-MMP, MT-MMP2	Gelatin, fibronectin, laminin, vibronectin, entactin, pro-MMP2
MMP 16	MT3-MMP, MT-MMP3	Collagen III, gelatin, fibronectin, casein, pro-MMP2
MMP 24	MT5-MMP, MT-MMP5	Fibronectin, gelatin, pro-MMP2, PGs
MMP 23 Unique structure (SA + TM + PP + CD (Zn) + Cys + Ig)		
MMP 23 A	MMP23A Ref here	Gelatin
MMP 23 B	MMP23B Ref here	Gelatin
SP= Signal peptide, PP = Propeptide, CD (Zn) = Catalytic domain containing Zinc, HPX = Hemopexin domain, Fur = Furin recognition motif, FN = Collagen binding type II motif of fibronectin		
GPI = Glycosylphosphatidylinositol anchor, TM = Trans-membrane domain, CT = Cytosolic tail, SA = amino-terminal signal anchor, Cys = Cystein rich domain, Ig = Immunoglobulin-like domain		

3. AIMS

The main aim of this thesis work was to investigate the role of S1P signaling and to elucidate the function of TRPC1 calcium channels in aggressive and highly metastatic human anaplastic and follicular thyroid cancer cells. More specifically, the goals of the studies included in this thesis work were to:

- I. Investigate the expression of S1P receptors and effects of S1P on human anaplastic thyroid cancer (ATC) cell migration and proliferation, and in part, to investigate the possible role of HERG potassium channels on migration in these cells.
- II. Identify the expression of TRPC channels in human follicular thyroid cancer ML-1 cells with emphasis on TRPC1 as modulator of migration and proliferation in these cells.
- III. Study the role of MMP2 and MMP9 in S1P-evoked attenuation of invasion of human anaplastic thyroid cancer C643 and follicular thyroid cancer FTC-133 cells.

4. MATERIALS AND METHODS

Materials

The materials used for this thesis research work are mentioned in the publications (I, II, III). All the chemicals and reagents used were of molecular biology and reagent grade.

Methods

The following methods were used to carry out experiments for this thesis research work; see publications (I, II, III).

4.1 Cell Culture

The cell lines and their respective culture medium conditions are mentioned in Table. 3. The cell cultures were maintained in a water-saturated atmosphere with 5% CO₂ and 95% air at 37C° in the incubators.

TABLE 3. The cell lines and their respective growth medium composition.

Cell line	Medium
Human follicular normal thyroid cell line N-thyr Ori 3-1 (II)	RPMI1640 supplemented with 10% FBS, 1% Penicilin/Streptomycin and 1% L-glutamine
Human anaplastic thyroid cancer cell line C643 (I, III)	DMEM supplemented with 10% FBS, 1% L-glutamine and 1% Penicilin/Streptomycin
Human anaplastic thyroid cancer cell line THJ-16T (I)	RPMI1640 supplemented with 10% FBS, 1% Penicilin/Streptomycin, 1mM sodium pyruvate and 25.03 mM HEPES
Human follicular thyroid cancer cell line FTC-133 (I, II, III)	DMEM : F12 Ham's (1:1), supplemented with 10% FBS, 1% L-glutamine and 1% Penicilin/Streptomycin
Human follicular thyroid cancer cell line ML-1 (I, II)	DMEM supplemented with 10% FBS, 1% L-glutamine and 1% Penicilin/Streptomycin
Human embryonic kidney cells (HEK-293) (II)	DMEM supplemented with 10% FBS, 1% L-glutamine and 1% Penicilin/Streptomycin
HEK-293 cells stably expressing HERG (HEK-HERG) in a pcDNA 3.1 vector (I)	DMEM supplemented with 10% FBS, 1% L-glutamine, 1% Penicilin/Streptomycin, and 0.2 mg/ml G418
Human immortal keratinocyte HaCat cells (II)	DMEM supplemented with 10% FBS, 1% L-glutamine and 1% Penicilin/Streptomycin
Rat normal thyroid FRTL-5 cells (II)	Coon's modified Ham's F-12 supplemented with 5% calf serum, 50 units/ml penicilin, and 50 µg/ml streptomycin and six hormones including 10 µg/ml insulin, 0.3 mIU/ml TSH, 10 nM hydrocortisone, 10 ng/ml somatostatin, 5 µg/ml transferrin and 10 ng/ml tripeptide Gly-His.Lys.

4.2 Viral transduction and generation of stable cell lines (II)

The cells were grown on 12-well plates and transduced with non-targeting shRNA lentiviral particles or TRPC1-targeting lentiviral particles according to the manufacturer's instructions (Sigma). After 48 hours, the medium was changed to fresh a medium containing 0.5 µg/ml puromycin. Here onwards, the cell cultures were maintained in the medium with 0.5 µg/ml puromycin. To check the knock-down

efficacy, TRPC1 expression was measured on mRNA and protein levels by quantitative PCR and western blotting, respectively.

4.3 Transient transfections (I, II, III)

Cells were transfected with siRNA or plasmid by using the electroporation method. Four million cells were pelleted and suspended in 400 μ l OptiMEM. The siRNA (2 μ M) or plasmid (20 μ g) was added and gently mixed by pipetting. The cells were electroporated at 975 microfarads and 240 V or 500 microfarads and 240 V as indicated. The transfected cells were grown for 48 hours and were then used for the experiments.

4.4 Qualitative and quantitative polymerase chain reaction (I, II, III)

The RNA was extracted with Aurum Total RNA minikit (I, II) or with TRI Reagent (III) according to the manufacturer's instructions. Human normal RNA, human papillary thyroid cancer RNA and human follicular thyroid cancer RNA were purchased as described elsewhere (II). RNA integrity was checked by absorbance spectroscopy and gel electrophoresis. RNA purity and concentrations were determined with Nanodrop 2000 (Thermo Fisher Scientific; Waltham, MA). cDNA was prepared with RevertAid reverse transcriptase (Thermo Fisher Scientific) from equal amounts of RNA. Reaction mixtures without reverse transcriptase or RNA were used as negative controls.

Qualitative and end point PCR - was performed with Jumpstat Taq DNA polymerase and a PTC-200 thermocycler. PCR products were run on agarose gels (containing ethidium bromide) and visualized under the UV light. MDA-MB-235, ML-1, HEK-293 and HaCat RNA were used as positive controls and hHPRT or β -Actin were used as reference genes.

Quantitative real time PCR assays Reaction mixtures were prepared with KAPA Probe Fast Master Mix (KAPA Biosystems; Boston, MA, USA). qRT-PCR was performed with the StepOnePlus Real Time PCR System (Applied Biosystems; Waltham, MA, USA) using the relative standard curve method.

Primers were designed with the Universal Probe Library (UPL) Assay Design Center (www.roccheapplied-science.com).

4.5 Western Blotting (I, II, III)

Cells were washed and extracted in ice-cold lysis buffer (Tris-base, 10 mM; NaCl, 150 mM; EDTA, 7 mM; NP-40, 0.5%; PMSF, 0.2 mM; pH 7.7) with a scraper. Protein concentrations were determined with the BCA protein assay reagent kit. Laemmli Sample Buffer (glycerol, 30%; SDS, 3%; Tris-HCl, 0.1875 M, pH 6.8; bromophenol blue, 0.015%; β -mercaptoethanol, 3%) was added to each extracted sample which was then boiled. Equal amounts of protein samples were subjected to SDS-PAGE (6-12%). After separation, the proteins were transferred to nitrocellulose membranes electrophoretically and were blocked for 1 hour with 5% non-fat dry milk in Tris-buffer saline with 0.1% Tween. The membranes were incubated with primary antibodies at 4°C overnight and with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Hsc70 and β -Actin were used as loading control. The membranes were incubated in ECL solution for 1 min and exposed to the films. Densitometric analysis was performed by using the Image J program (National Institute of Health, Bethesda, MD, USA). The results were corrected for protein loading by normalization with Hsc70 or β -Actin expression.

In experiments for measuring MMP2 and MMP9 expression, the lysis buffer had no PMSF. Similarly, the boiling of the samples after addition of LSB was omitted because MMP2 and MMP9 are sensitive to heat.

In experiments for measuring the secretion of MMP2 and MMP9, the cells were treated as mentioned elsewhere (II, III). The medium was collected and the loading buffer was added. Equal volumes of medium were loaded on the gels and the western blotting protocol was followed as described above.

4.6 Migration or Invasion Assays (I, II, III)

Migration or invasion assays were performed on 6.5 mm diameter Transwell inserts with 8 μ m pore size. For the invasion assays, the membranes were coated with 5 μ g/cm² collagen I or collagen IV and allowed to dry overnight in the laminar. Next day, the membranes were reconstituted with serum-free medium (SFM) for 1 hour at 37°C. In some experiments, the cells were serum starved overnight and then pre-incubated with antagonists, inhibitors or activators for the indicated time. Cells in SFM were added to the upper wells. Lipid striped fetal bovine serum 5 -10% (LS-FBS) was used as chemoattractant with or without S1P. The inhibitors or antagonists were present in both

the upper and lower wells. Cells were allowed to migrate for 6 or 16 hours. The non-migrated cells on top of the membranes were wiped with a cotton swab. The migrated cells were fixed in 2% paraformaldehyde (PFA) for 10 min and stained with 0.1 crystal violet in 20% methanol for 5 min. The membranes were rinsed with PBS and water and allowed to dry overnight. The cells were counted at 40X magnification in a straight line bisecting the membrane.

4.7 Wound Healing Assays (III)

Cells were grown to 90% confluency and serum starved overnight. Then 0.5 $\mu\text{g/ml}$ mitomycin C was added to inhibit cell proliferation. The monolayer of cells was scratched with a micropipette tip (200 μl). The cells were treated with 100 nM S1P, while vehicle was added to the control plates. The images were taken immediately (0 h) and after (24 h) with a Leica microsystems camera and framework software (Leica microsystems; Wetzlar, Germany). The distance travelled by the cells to close the wound was measured. The results were presented as % wound.

4.8 Zymography (II, III)

Cells were grown up to 80-90% confluency. Cells were treated as indicated and the medium was collected. Equal volumes of sample medium were mixed with loading buffer. The samples were electrophoresed on 10% SDS gels containing 2.65 mg/ml gelatin. Next, the gels were incubated in zymo-buffer I for 30 min. The gels were then incubated in Zymo II buffer for 30 min. For gelatinolytic activity, gels were incubated at 37°C overnight in buffer III. The gels were visualized under UV light for protein ladder marker and after that the gels were stained with Comassi Blue R250 for 1-2 hours. The gelatinolytic activity was visualized as clear bands against blue background on stained gels. The bands were quantified with the Image J program. The data were normalized with the respective total protein concentrations of the cells on the culture plates.

TABLE 4. Zymography Buffers composition.

Buffer	Composition
Loading Buffer	0.1 M Tris-phosphate buffer (pH 6.8) containing 20% glycerol, 6% SDS, 0.04% bromophenol blue
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

4.9 Proliferation Assays (I, II)

The [^3H] thymidine incorporation method was used to study the proliferation of the cells. 75,000-100,000 cells were grown on 35-mm plates for 24, 48 and 72 hours. Four hours prior to the end of each experiment, 0.4 $\mu\text{Ci/ml}$ [^3H] thymidine was added. The cells were washed three times with PBS, incubated for 10 min with trichloric acetic acid (TCA) and then incubated for 10 min with 0.1 M NaOH. The radioactivity was measured using a Wallac 1410 liquid scintillation counter.

4.10 Fluorescence-activated cell sorting (FACS) analysis (II)

500,000 cells were grown on 35-mm plates overnight. The cells were detached and pelleted by centrifuging. The pellets were suspended in 500 μl of propidium iodide (PI) solution (0.05 mg/ml propidium iodide, 3.8 μM sodium citrate and 0.1% Triton X-100 in PBS). The suspended cells in PI solution were incubated at room temperature for 15 min in the dark. The samples were then analyzed by flow cytometry using FACS Calibur and CellQuest Pro software. The percentage of cells in each phase of the cell cycle was calculated by using ModFit LT 4.1 software.

4.11 Intracellular Calcium Measurements (II)

4.11.1 Cell Suspension (II)

The medium was aspirated and the FRTL-5 cells were harvested with Hank's balanced salt solution buffer (HBSS) lacking calcium but containing 0.02% EDTA and trypsin. The cells were washed three times and incubated with 2 μM Fura 2-AM in HBSS for 30 min at room temperature. The cells were washed with HBSS, incubated for 10 min at room temperature and then washed once more. Fluorescence was measured at 37°C with a Hitachi F2000 fluorimeter using excitation wavelengths of 340 and 380 nm and emission was detected at 510 nm. The signal was calibrated by addition of 1 mM CaCl_2 and Triton X-100 (maximum fluorescence), and then chelation of extracellular calcium with 5 mM EGTA and addition of Tris-base to elevate the pH above 8.3 (minimum fluorescence). Intracellular calcium $[\text{Ca}^{2+}]_i$, was calculated as described (Grynkiewicz et al. 1985), using a K_d value of 224 nM for Fura-2.

4.11.2 Adherent Single Cell Imaging (II)

Cells cultured on poly-L-lysine coated coverslips were washed twice with HBSS and incubated with 2 μ M Fura-2 AM for 30 min at room temperature in the dark. The cells were then washed with HBSS. The coverslip was placed in a perfusion chamber that was mounted on a Zeiss Axiovert 35 microscope. The 340 nm and 380 nm excitation filters were used and emission was measured at 510 nm. Light was obtained from an XBO 75W/75 xenon lamp. The shutter was controlled by a Lambda 10-2 control device (Sutter Instruments, Novato, CA, USA). A HAMAMATSU digital camera C10600 ORCA-R² with controller (Photronics K.K.) was used to capture fluorescence images at 1-3 seconds to avoid bleaching. Images were processed using the Axon Imaging Workbench 6 software (INDEC BioSystems, Santa Clara, CA). The experiments were performed at room temperature. For experiments in calcium free HBSS, 150 μ M EGTA was added but no CaCl₂. The F_{340} / F_{380} ratio was used as a measure of intracellular free calcium concentrations.

4.12 Immunocytochemistry (I)

Cells were grown on poly-L-lysine-coated glass coverslips on 35-mm plates for 24 h. The next day, the cells were serum starved for 24 h. The cells were then stimulated with 100 nM S1P for 0, 30, and 60 min. The cells were washed three times with PBS and fixed with methanol-acetic acid (95:5) for 5 min at -70 C°. After fixation, the cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were washed three times with PBS and blocked with 5% normal goat serum in PBS for 60 min. Cells were incubated with the anti-HERG antibody (1:100) overnight at +4 C°. The next day, after three washes with ice-cold PBS, the cells were re-blocked with 5% normal goat serum in PBS for 60 min and treated with the secondary antibody (Alexa Flour-568 goat anti-rabbit antibody, 1:500) for 1 h. In negative controls, the cells were only incubated with the secondary antibody. The slides were mounted with Mowiol mounting medium containing DAPI (1.5 mg/ml). Confocal images were obtained using a Zeiss LSM 780 laser scanning confocal microscope (Jena, Germany). Images were taken using a 63x 1.2 water objective. The images were acquired with ZEN 2010 software.

4.13 Rac activation assays (I)

Cells (50,000) were plated on 35-mm plates, grown until they reached 50% confluency, and serum-starved for 24 h. The cells were treated with S1P 100 nM for 0, 1, 3, 6, 12, and 30 min and lysates were made. The samples were aliquoted for protein concentration measurement, and the rest were snap-frozen immediately after harvesting and clarification as directed in the user's manual. The protein concentration was measured using the BCA kit. The protein concentration used for assays was kept at 0.5 mg/ml by equalization of protein concentration in each sample. The Rac activation assay was performed by using a Rac 1,2,3 G-LISA Activation Assay kit (Cytoskeleton, Inc. Denver, CO, USA) according to the manufacturer's instructions.

4.14 Rho activation assays (III)

Cells (50,000) were plated on 35-mm plates, grown until they reached 50% confluency, and then serum-starved for 24 h. The cells were treated with 100 nM S1P for 0, 1, 3, 6 and 12 min and the lysates were made. The samples were aliquoted for protein concentration measurement, and the rest were snap-frozen immediately after harvesting and clarification as directed in the user's manual. Protein concentration was measured using the BCA kit. The protein concentration used for assays was kept at 0.5 mg/ml in each sample. Rho activation was measured with a G-LISA Rho activation assay kit (Cytoskeleton, Inc., Denver, CO, USA) according to the manufacturer's instructions.

4.15 Calpain Activity Assays (III)

Two million cells were grown on 100-mm plates. The cells were serum-starved overnight and treated with 100 nM S1P for 6 h. The cells were detached and washed three times with PBS. Thereafter, calpain activity assays were processed according to the manufacturer's instructions (Abcam, Cambridge, MA, USA). Fluorescence was measured by a Hidex sense microplate reader instrument (HIDEX Corp, Turku, Finland) with excitation at 400 nm and emission at 505 nm. The protein concentration was kept at 60 mg/ml for each sample.

4.16 Patch clamp studies (I)

C643 cells and HEK-HERG cells grown as described elsewhere (I), and plated at low density onto round glass coverslips kept in 24-well plates. Cells were grown in SFM (0.2%

FAF-BSA) for 24-48 hours. The C643 cells were incubated with the extracellular solution (ECS) described in Table 5. The used intracellular solution (ICS) in the patch pipettes is also shown in Table 5. In HEK-HERG recordings, the ECS and ICS composition was kept as mentioned in the Table. Whole-cell currents were recorded as described by (Hamill et al. 1981), at 22–24 °C using an EPC-9 amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). Series resistance was at least 80% compensated for and currents were sampled at 5–10 kHz and filtered at 1–2 kHz. Pipettes pulled from thin walled borosilicate glass (Harvard Apparatus Ltd, Edenbridge, UK) had a resistance of 3–6 MU when filled with above-mentioned ICSs. The voltage-pulse protocol used was constituted from an 800 ms step to +40 mV that was followed by a series of 200 ms rectangular pulses from +10 to -130 mV at 10 mV decrements and was used to construct the IV profile of HERG tail currents. Cells were held at -70 mV between pulses. The above-mentioned voltage values were corrected off-line for a liquid junction potential (LJP) of 17 mV in C643 experiments and for a LJP of 5 mV in HEK-HERG experiments. LJP values were estimated with the JPCalc software (Barry 1994). Data analysis and illustrations were done with PulseFit (HEKA Elektronik) and Origin (OriginLab, Northampton, MA, USA) software. Leak current estimated with a P/4 protocol at -70 mV was subtracted after which the peak tail currents were detected.

TABLE 5. Composition of Patch-clamp solutions

ECS (C643)	150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl ₂ , 1.2 mM MgCl ₂ , 5 mM HEPES, pH 7.4, and 100 mM NaCl, 55.5 mM KCl, 1.8 mM CaCl ₂ , 1.2 mM MgCl ₂ , and 5 mM HEPES, pH 7.4
ICS (C643)	135 mM KGluc, 5 mM MgATP, 5 mM BAPTA, 2 mM MgCl ₂ , and 10 mM HEPES, pH 7.2
ECS (HEK-HERG)	150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl ₂ , 1.2 mM MgCl ₂ , and 5 mM HEPES, pH 7.4 (supplemented with 1 mg/ml FAF-BSA)
ICS (HEK-HERG)	135 mM KCl, 5 mM MgATP, 5 mM BAPTA, 2 mM MgCl ₂ , and 10 mM HEPES, pH 7.2

4.17 Statistics (I, II, III)

Results are presented as the mean \pm SEM from at least three independent measurements. Student's *t*-test was applied to the data when two means were compared. One-way ANOVA and Bonferroni's *post hoc* test was used when three or more means were compared. The statistical significance of patch-clamp data was tested with nonpaired student's *t*-test. The GraphPad Prism 5 software (GraphPad Software Inc.; San Diego, CA) was used for statistical analyses. P-values < 0.05 were considered statistically significant.

5. RESULTS AND DISCUSSION

5.1 Publication I.

This publication focuses on the expression of S1P receptors and the importance of S1P to regulate migration in human anaplastic thyroid cancer C643, THJ-16T and follicular thyroid cancer FTC-133 cells. We show for the first time that S1P1-3 are expressed and S1P attenuates migration in these cells. This inhibitory effect of S1P is mediated by S1P2 and downstream activation of the Rho-ROCK pathway. In addition, we show that the HERG channels are expressed as non-conducting ion channels in C643 cells. S1P transiently attenuates the expression of HERG channel and, in part, the migration in C643 and HEK-HERG⁺ cells.

S1P receptor expression has been shown previously in thyroid cancer cells, but the identity of some cancer cells was unclear (Balthasar et al. 2006). Therefore, in the present study we have used C643, THJ-16T, ML-1 and FTC-133 cell lines which are the verified human thyroid cancer cells (Schweppe et al. 2008; Marlow et al. 2010).

5.1.1 Anaplastic human thyroid cancer cells express S1P receptors

S1P acts through S1P receptors to regulate cell migration and proliferation in normal and cancer cells (Pyne & Pyne 2010). We show for the first time that S1P1-3 are expressed both on mRNA and protein levels in anaplastic thyroid cancer C643 and THJ-16T cells (I. Figs. 1A and 1B). This S1P receptor expression profile is the same as previously reported for human primary thyroid cells and follicular thyroid cancer ML-1 and FTC-133 cells (Balthasar et al. 2006; Bergelin et al. 2009). The HEK-HERG⁺ cells also express S1P1-3 (Table 6 below). Despite the same receptor profile, S1P stimulates migration in follicular thyroid cancer ML-1 cells (Balthasar et al. 2006). Interestingly, S1P is without an effect on the primary thyroid and N-thyr Ori 3-1 cell migration (Balthasar et al. 2006; Asghar and Törnquist unpublished observation). However, S1P attenuates migration in C643, THJ-16T, FTC-133 and HEK-HERG⁺ cells (II. Figs. 2A and 8D). S1P1 and S1P3 have been shown to participate in promoting migration, while S1P2 has been observed as an anti-migratory receptor (Balthasar et al. 2006). These findings clearly suggest that the S1P receptor profile per se cannot be used to define the migratory potential of the normal and thyroid cancer cells. In fact, it is the specific downstream signaling pathway activation which decides the migratory fate of these cells.

TABLE 6. Expression of S1P1-3 receptors in normal and thyroid cancer cells.

	S1P1	S1P2	S1P3
C643	+	+	+
THJ-16T	+	+	+
FTC-133	+	+	+
HEK-HERG+	+	+	+
ML-1	+	+	+
Primary Thyroid	+	+	+
N-Thyr-Ori 3-1	+	+	+

(Red, pro-migratory receptors; Green, anti-migratory receptor)

5.1.2 Effects of S1P on thyroid cancer cell proliferation and migration.

We found that the S1P is without an effect on proliferation of C643, THJ-16T and FTC-133 cells (I. Fig. 3). Why S1P has no effect on proliferation in these cells is an open question. We have no explanation for this now. Previously, we have reported that S1P is without an effect on proliferation of primary thyroid cells (Balthasar et al. 2006). In addition, S1P was unable to affect proliferation of N-thy-Ori 3-1 cells (Asghar and Törnquist unpublished observation). However, S1P was able to decrease proliferation slightly but significantly in ML-1 cells (Balthasar et al. 2006).

S1P potently attenuated migration in C643, THJ-16T and FTC-133 cells (I. Fig. 2A). This contrasts with the effect of S1P in ML-1 cells, where S1P stimulates migration by S1P1,3, which activate the downstream PI3K-Akt pathway (Balthasar et al. 2006). We characterized the mechanism by which S1P inhibits migration in C643 cells. Using the S1P2 antagonist JTE-013, the S1P1,3 antagonist VPC 23019 and S1P2 siRNA, we showed that the inhibitory effect of S1P was mediated by S1P2 and not by S1P1,3 (I. Figs. 2B-2D).

S1P2 couples to G12/13 strongly and activates the Rho and ROCK pathway, which results in inhibition of tumor cell migration (Okamoto et al. 2000; Takashima et al. 2008; Takuwa et al. 2011; Lepley et al. 2005; Witt et al. 2012). We show that the inhibition of Rho and ROCK with the inhibitors C3 transferase and Y-27632, respectively, abolishes the inhibitory effect of S1P on C643 cell migration (I. Figs. 4A and 4B). Previously, it has been reported that in Chinese hamster ovary (CHO) cells, PI3K-dependent activation of Rac is strongly inhibited by activation of Rho, which leads to a decrease in migration (Yamaguchi et al. 2003). In addition, S1P has been shown to inhibit the migration of mouse melanoma B16F10 cells by activation of Rho and subsequent inhibition of Rac. However, this effect was dose dependent (Yamaguchi et al. 2003). In our human

anaplastic thyroid C643 cancer cells, we show that migration was attenuated by blocking Rac, and that the Rac activity was transiently attenuated by S1P (I. Figs. 4C and D). Furthermore, S1P potently stimulates Rho activity in C643 cells (III. Fig. 5A).

5.1.3 HERG Potassium channels regulate migration and proliferation.

HERG potassium channels, also known as Kv11.1, regulate cancer cell migration and proliferation (Arcangeli 2005; Prevarskaya et al. 2010; Arcangeli & Becchetti 2015). We show that HERG potassium channels are expressed in C643 cells, both on mRNA and protein levels (I. Figs. 6 A and B). In C643 cells, no HERG tail currents were detected (I. Fig. 6F) as compared to the HEK293 HERG⁺ overexpressing cells, which exhibit massive tail currents (I. Fig. 8C). This suggests that HERG channels in C643 cells are present in a non-conducting form. Previously, it has been shown that non-conducting forms of HERG channels do participate in cellular functions (Kaczmarek 2006). C643 cells, pre-incubated with the HERG inhibitor E-4031, showed that the basal migration was attenuated and that S1P enhanced the effect of E-4031 (I. Fig. 6C). Previously, the Törnquist lab observed that HERG⁺ inhibition attenuated migration in ML-1 cells (Bergelin and Törnquist, unpublished observations) and in HEK-HERG⁺ cells (Afrasiabi et al. 2010).

In C643 cells stimulated with S1P, the HERG protein expression was transiently decreased (I. Figs. 7A and 7C). As control, we used HEK-HERG⁺ cells and observed that S1P transiently decreased the expression of HERG and attenuated the migration in these cells (I. Figs. 8A, 8B and 8D). We speculate that this S1P-evoked transient decrease in HERG expression is due to the S1P-evoked production of diacylglycerol (DAG) and subsequent internalization and degradation of HERG channel protein. We showed previously that in HEK-HERG⁺ cells, DAG induced a transient decrease in HERG protein expression (Ramström et al. 2010). However, this possibility was not investigated here. Thus, the HERG potassium channels are involved, at least in part, in regulating C643 and HEK-HERG cell migration.

We conclude that S1P1-3 are expressed in normal and thyroid cancer cells. S1P attenuates migration by S1P2 and through downstream Rho and ROCK pathway activation in C643 cells. In part, this S1P-evoked inhibition of migration is due to the transient decreased in the HERG⁺ channel protein.

5.2 Publication II.

5.2.1 *TRPC channels are expressed in human thyroid tissue and cell lines*

Calcium is essential for thyroid function, but the expression of calcium channels remained elusive (Törnquist et al. 1997). In the present study, we show for the first time that several TRPC channels are expressed in both the human normal thyroid tissue, and the normal thyroid cell line N-thyr-ori 3-1, in follicular thyroid cancer tissue and in the follicular thyroid cancer cell lines ML-1 and FTC-133, as well as in papillary thyroid cancer tissue (II. Fig. 1A). We decided to investigate TRPC1 in this study, as TRPC1 has been shown to be ubiquitously expressed in humans and to regulate many cellular processes, including cell migration and proliferation in both normal and cancer cells (Riccio et al. 2002; Abramowitz & Birnbaumer 2009; Kuang et al. 2012; Nesin & Tsiokas 2014). To study the importance of TRPC1 in follicular thyroid cancer ML-1 cells, we generated a stable TRPC1 knock-down ML-1 cell line (TRPC1-KD).

5.2.2 *S1P-evoked calcium entry is attenuated in TRPC1-knockdown cells*

A multitude of reports have shown that TRPC1 is important in store-operated calcium entry (SOCE) (Wes et al. 1995; Ambudkar 2007; Cheng et al. 2013). We investigated if TRPC1 was involved in SOCE in ML-1 cells. However, we could not observe a significant difference in the thapsigargin (Tg) evoked calcium increase or in calcium entry when calcium was re-added to Tg-treated TRPC1-KD cells compared with the control MOCK-transfected ML-1 cells (II. Figs. 1C-1F). TRPC1 has been shown to evoke receptor-operated calcium entry (ROCE), as well (Nesin & Tsiokas 2014; Storch et al. 2012). In calcium free conditions, S1P was unable to induce a change in intracellular calcium in TRPC1-KD cells or in the MOCK transfected cells. Interestingly, in the presence of calcium, the S1P-evoked calcium response was markedly attenuated in TRPC1-KD cells, compared with the MOCK transfected cells (II. Figs. 1J and 1K). This suggests that knockdown of TRPC1 did not affect SOCE but had a strong effect on ROCE in ML-1 cells. Similar results were observed in TRPC2 knock-down rat thyroid FRTL5 cells (II. Fig. 2C), where TRPC2 is the only expressed TRPC channel and functions as a ROCE channel (Törnquist et al. 2014). Thus, in ML-1 and FRTL-5 cells, S1P activates calcium entry through TRPC1 and TRPC2, respectively. Previously, it has been reported that S1P upregulates the expression of TRPC1 and concentrates it in the membrane micro-domains during mouse myoblast differentiation in mouse C2C12 cells (Formigli et al. 2009). In addition, S1P has been shown to activate TRPC6 calcium channels in

astrocytes, inducing calcium influx and activation of the MAPK pathway and the secretion of the neuroprotective chemokine CXCL1 (Shirakawa et al. 2017b). Furthermore, in vascular smooth muscle cells, TRPC5 is activated by S1P, resulting in influx of calcium into the cells and activation of migration of these cells (Xu et al. 2006). Thus, S1P activates TRPC channels and the resulting calcium influx regulates different cellular processes.

5.2.3 TRPC1-knockdown attenuates the migration and the expression of pro-migratory receptors in thyroid cancer cells

TRPC channels, including TRPC1, are important regulators of cancer cell migration (Schwab et al. 2012; Nesin & Tsiokas 2014). Previously, it has been reported that S1P enhances migration in ML-1 cells by activating S1P_{1,3} and through a cross-talk with VEGFR2 (Balthasar et al. 2006; Balthasar et al. 2008; Bergelin et al. 2010). In TRPC1-KD ML-1 and FTC-133-KD cells, we observed a decrease in basal as well as in S1P-evoked migration compared to MOCK cells (II. Figs. 2A, 3A and 3B). Similarly, there was a decrease in both basal and S1P-evoked migration in TRPC2-KD FRTL-5 cells (II. Fig. 2B). Interestingly, in the TRPC1-KD cells, S1P₃ and VEGFR2 were significantly downregulated (II. Figs. 3C and 3D). To confirm this finding, wild type ML-1 cells were transfected with either TRPC1 siRNA or with a dominant negative pore mutated TRPC1, or incubated with a TRPC1 specific toxin GsMTx-4. In these cells, both the expression of S1P₃ and VEGFR2 was significantly attenuated (II. Figs. 4B, 4C, 4E, and 4F). However, transfecting back TRPC1 in TRPC1-KD cells restored not only the expression of S1P₃ and VEGFR2 but also the basal and S1P-evoked migration (II. Figs. 5A-5D). Furthermore, in ML-1 cells pre-incubated with BAPTA or calmodulin and calmodulin kinase inhibitors, the S1P₃ and VEGFR2 expression and migration were significantly attenuated (II. Figs. 6A-6H). Thus, calcium regulates the expression of pro-migratory S1P₃ and VEGFR2 receptors and invasion and migration of ML-1 cells.

Previously in our laboratory, S1P₃ has been shown to regulate the expression of VEGFR2, HIF1 α , MMP2 and MMP9 in ML-1 cells. S1P increases HIF1 α activity and HIF1 α mediates S1P-induced migration. The migration evoked by S1P_{1,3} is mediated in part by activating MMP2 and MMP9 (Bergelin et al. 2010; Kalhori et al. 2013; Kalhori & Törnquist 2015). In this study, we show that, in TRPC1-KD cells, due to less calcium, there was a decreased expression of HIF1 α and that the secretion and activity of MMP2 and -9 were significantly decreased, compared with MOCK cells (II. Figs. 7A-7E). Similarly, in rat FRTL-5 cells,

TRPC2-KD resulted in decreased expression of S1P3 and subsequent decrease in the secretion of MMP2 and S1P-evoked migration (Sukumaran et al. 2013). At present, we do not know by which mechanism exactly S1P3 regulates VEGFR2. However, we found that in TRPC1-KD cells the expression of PKC β , PKC δ and ERK1/2 was attenuated (II. Figs. 8A-8D). These kinases have been shown to be involved in S1P3-induced expression of both VEGFR2 and HIF1 α in ML-1 cells (Bergelin et al. 2010; Kalhori et al. 2013). Previously, it has been shown that S1P enhances invasion of epithelial ovarian cancer cells by activation of MMP2 and associated degradation of the ECM. This activation of MMP2 by S1P is Gi and Rac mediated. Also, in breast cancer cells, S1P induces the invasion by increased MMP9 secretion and through S1P3-G α q coupling (Kim et al. 2011). These reports highlight the association of S1P signaling with MMP effects.

5.2.4 Effects of TRPC1 knockdown on ML-1 cancer cell proliferation

TRPC1 has been shown to participate in normal as well as cancer cell proliferation (Kuang et al. 2012; Nesin & Tsiokas 2014). The present results show that the proliferation was significantly downregulated in TRPC1-KD cells compared to the MOCK cells (II. Fig. 9A). To strengthen the evidence, ML-1 wild type cells were transfected with a dominant negative, pore-mutated, nonconducting TRPC1 channel or with TRPC1 siRNA. The results show that in both conditions, the proliferation was significantly decreased, which advocates the significance of TRPC1 in proliferation of ML-1 cells. In addition, when the TRPC1 was transfected back in TRPC1-KD cells, proliferation was restored. Thus, TRPC1 is important in regulating thyroid cancer cell proliferation (II. Figs. 9B-9D). Furthermore, FACS analysis of the cell cycle showed that there was a significantly higher number of cells in the G1 phase, and that the S and G2 phases were significantly decreased in TRPC1-KD cells, compared to MOCK transfected cells. The expression of the cell cycle regulatory proteins p21^{kip1} and p27^{waf1/cip1} was significantly increased in TRPC1-KD cells, whereas the expression of cyclin D2, cyclin D3 and cdk6 was significantly attenuated (II. Figs. 10A-10F).

We conclude that TRPC1 channels in ML-1 cells function as ROCE channels and not as SOCE channels and the downregulation of TRPC1 channels affects cell migration and proliferation, in part, by downregulating the pro-migratory receptors S1P3 and VEGFR2 and the downstream signaling molecules in a calcium-dependent manner. This work is a novel contribution to better understand the etiology of thyroid cancer and to design new methods and strategies to find effective treatments for thyroid cancer.

5.3 Publication III

S1P evokes thyroid cancer ML-1 cell migration through S1P1,3 receptors and by the activation of PI3K/Akt and Rac migratory pathways. This effect is in part mediated by the enhanced secretion of MMP2 and MMP9 (Gonda et al. 1999; Windh et al. 1999; Okamoto et al. 2000; Ishii et al. 2002; Taha et al. 2004; Balthasar et al. 2006). On the contrary, S1P has been shown to inhibit cell migration through S1P2 receptor and downstream activation of Rho and ROCK pathways (Ishii et al. 2002; Hashimoto et al. 2008; Takuwa et al. 2011; Witt et al. 2012). In this study, we have investigated the role of MMP2 and -9 in ATC C643 cell invasion and migration. The results show that S1P through S1P2 attenuates the expression, secretion and activity of MMP2 and to a lesser extent, the secretion and activity of MMP9. In addition, S1P regulates calpain activity by S1P2. These findings describe a novel role for S1P2 and present a new mechanism by which S1P inhibits migration of C643 cells.

5.3.1 S1P-evoked inhibition of MMP2 and MMP9 expression, secretion and activity in C643 cells

MMPs, the largest group of extracellular matrix degrading proteolytic enzymes, are indicated in promoting invasiveness of several malignant forms of cancers (Devine et al. 2008; Kim et al. 2011). Previously, MMP2 and MMP9 have been shown to actively participate in the thyroid cancer cell invasion (Rajoria et al. 2011; Zhang et al. 2012; Yang et al. 2013; Kalhori & Törnquist 2015). We have shown recently that S1P promotes invasion and migration of ML-1 cells in part, by S1P1,3 induced increase in secretion and activity of MMP2 and MMP9 (Kalhori & Törnquist 2015). We determined the effect of S1P on MMP2 and MMP9 expression, secretion and activity in C643 cells (III. Figs. 1A,1C and 1D). In C643 cells, incubated with S1P for the indicated times, the expression, secretion and activity of MMP2 were significantly attenuated. (III. Figs 1A, 1C and 1D). However, a dual response to S1P was observed on MMP9 expression. As can be seen in (III, Fig. 1A), S1P decreased the expression of MMP9 initially at 2 h and 4 h, but significantly enhanced it at 6h and caused no significant change at 8h. These results were in line with the recent report that S1P enhances the expression of MMP9 by S1P2 (Bi et al. 2014; Kalhori & Törnquist 2015).

5.3.2 S1P-evoked inhibition of MMP2 and MMP9 expression, secretion and activity in FTC-133 cells

As a control cell line, we used human follicular thyroid cancer FTC-133 cells. S1P decreased the expression, secretion and activity of MMP2 at 6 h and 8 h in human

follicular thyroid cancer FTC-133 cells (Figs. 2A, 2B and 2C). In addition, the invasion of FTC-133 was also decreased both at 6 h and 8 h (Fig.2D). Interestingly, in FTC-133 cells S1P decreased the expression, secretion and activity of MMP9 at 6 h but not at 8 h. The expression of MMP9 was significantly increased at 8 h, had no effect on secretion but activity decreased both at 6 h and 8 h, as can be seen in Figs. 2A-2D.

5.3.2 S1P-evoked inhibitory effect of MMP2 expression, secretion and activity is mediated by S1P2.

S1P inhibits migration of C643 cells through activation of S1P2 (Asghar et al. 2012). Thus, we wanted to identify the S1P receptor, which mediates the S1P-evoked decrease in expression, and activity of MMP2 in these cells. By using the S1P1,3 antagonist VPC-23019, the S1P2 antagonist JTE-013 and S1P2 siRNA, we could show that the S1P-evoked attenuation of MMP2 expression and activity was mediated by S1P2 and not by S1P1,3 (III. Figs. 3B, 3C, 3E and 3F). Thus, we showed here that in C643 cells, S1P2 has an inhibitory effect on MMP2 expression, secretion and activity. Interestingly, in contrast to our observation, S1P2 has been shown to promote the production of MMP9 and migration in pancreatic stellate (PSCs) cells (Bi et al. 2014).

5.3.3 MMP2 and MMP9 participate in C643 cell invasion

After observing the S1P-evoked effects on expression, secretion and activity patterns of MMP2 and -9, we investigated their impact on cell invasion. As can be seen in (III. Fig. 1E), S1P potently attenuated invasion at all time points, and this inhibition was mediated by S1P2 (III. Fig. 4A). In C643 cells, by downregulating MMP2 and MMP9 with siRNA or by blocking them with pharmacological inhibitors, the basal invasion was significantly attenuated. However, S1P was still able to decrease invasion slightly but significantly. This clearly suggests that MMP2 and -9 are not the only players in S1P-evoked inhibition of C643 cell invasion, but also other signaling pathways are involved (III. Figs. 4B-4E).

5.3.4 S1P regulates calpain activity by S1P2 and mediates S1P-evoked effects of MMP2 and -9

Calpains are calcium-dependent endopeptidases which cleave inactive pro-MMPs to active MMPs and regulate many cellular functions including cell invasion (Storr et al. 2011; Potz et al. 2016). S1P has been shown previously to regulate calpain activity in human umbilical vein endothelial (HUVE) cells, and calpain inhibition decreased S1P-evoked invasion in these cells (Kang et al. 2011). In thyroid cancer ML-1 cells, calpains

participate in S1P-evoked invasion and MMP2 and MMP9 secretion (Kalhori & Törnquist 2015). Our results showed that in C643 cells, S1P decreased calpain activity and by using S1P2 siRNA, we found that this decrease was mediated through S1P2. We used a pharmacological approach and blocked calpains with a potent inhibitor, ALLN. The results showed that by blocking calpains in C643 cells, the expression of MMP2 and -9 and the secretion of MMP2 were attenuated, which resulted in attenuated cell invasion (III. Figs. 5A-5E).

5.3.5 S1P-evoked inhibition of MMP2 is mediated through Rho and ROCK pathway

Upon activation S1P2 preferentially couples to G12/13 and activates the small GTPase Rho. Active Rho inhibits Rac and results in inhibition of cell invasion (Okamoto et al. 2000; Lepley et al. 2005; Takashima et al. 2008). In the present study, we show that S1P increased Rho activity in C643 cells (III. Fig. 6A). Furthermore, when these cells were stimulated with S1P, the Rac activity was transiently attenuated (I. Fig. 4D). In addition, blocking Rac with a pharmacological inhibitor resulted in attenuated C643 cell invasion (I, Fig. 4C). Rac is required for MMP2 activation in HT1080 fibrosarcoma cells (Zhuge & Xu 2001). This suggests that the S1P-evoked Rho activation and inhibition of Rac in C643 cells, in part, decreases activation of MMP2. By using Rho and ROCK inhibitors, we showed that the basal invasion was slightly but significantly enhanced in C643 cells. These findings are in line with results showing that Rho or ROCK inhibition enhances migration towards serum in mesenchymal stromal cells (Jaganathan et al. 2007). As can be seen in III, Figs. 6C-6E, in C643 cells treated with either Rho or ROCK inhibitors, there was no change in basal MMP2 expression, but the S1P-evoked inhibition of MMP2 expression and activity was abolished. Thus, S1P through S1P2 and activation of Rho and ROCK pathways regulates the inhibition of MMP2.

We conclude that S1P regulates the expression, secretion and activity of MMP2 and MMP9 in C643 cells. This, at least in part, attenuates invasion in these cells. We present here a novel mechanism by which S1P, by activating S1P2, can attenuate invasion and migration of cancer cells. This study highlights the importance of the S1P2 receptor in regulating thyroid cancer cell invasion and migration.

6. CONCLUDING REMARKS

Based on the results of the three projects conducted for this thesis research work, we conclude:

1. S1P attenuates migration in ATC C643 cells, by activating S1P2 and the Rho and ROCK pathway, and in part, through downregulation of HERG protein.
2. Several TRPC channel isoforms are expressed in both thyroid tissue and cell lines.
3. TRPC1 functions as a ROCE channel in follicular thyroid cancer ML-1 cells and knockdown of TRPC1 results in the attenuated proliferation and migration.
4. TRPC1 has an important role in the regulation of the pro-migratory receptors S1P3 and VEGFR2 by a calcium-dependent mechanism in ML-1 cells.
5. S1P attenuates the expression, secretion and activity of MMP2 and to a lesser extent MMP9. The inhibitory effect of S1P on MMP2 expression and secretion is mediated by S1P2 through the Rho and ROCK pathway, and in part, by decreasing calpain activity.

This thesis work highlights the importance of S1P signalling and calcium signalling in thyroid cancer cell proliferation and migration. S1P has been found to have dual and opposite actions on human thyroid cancer cell migration. It is suggested that the functional outcome of S1P depends not only on the expression of S1P receptors but also on the downstream signalling pathway it activates. Thus, S1P and S1P receptors have emerged as potential targets for drug development. Currently, no effective treatment is available against aggressive and highly invasive thyroid cancer cells. However, the S1P1 receptor has been therapeutically targeted for treating multiple sclerosis by Fingolimod (FTY720), which is an immunosuppressive drug approved by US Food and Drug Administration (FDA). Recently, our *in vitro* findings have suggested that FTY720 is a potent drug of choice to treat thyroid cancer. In this thesis, we also present S1P2 signalling as a powerful strategy for the development of new drugs for the treatment of thyroid cancer. Recently, a novel anti-S1P antibody approach has been adopted to cure malignant renal cancer. The same approach could be used for thyroid cancer cells in which S1P promotes migration. However, a reverse strategy, *i.e.* overexpression of S1P2 and injection of S1P, or the use of a selective S1P2 agonist, could help in treating anaplastic thyroid cancer where S1P inhibits migration. Previously, S1P has been shown

to inhibit the formation of lung metastasis of B16/F10 cells and S1P2 overexpression enhanced this effect. On the other hand, several reports have indicated the pro-tumorigenic role of S1P2. Thus, the selection of the right strategy is essential in the designing and development of drugs targeting S1P and S1P receptors.

HERG channels are present in many normal cells, including cardiac and smooth muscle cells. HERG channels are primary targets in the treatment of arrhythmias. HERG is overexpressed in tumour cells and is associated with a more aggressive phenotype. Apparently, the inhibition of HERG channels appears as very promising strategy. But as a serious side effect, many drugs are found to induce arrhythmias and sudden death. To be able to use HERG channels on tumours as drug targets, a highly tissue specific, targeted approach is essential for the successful treatment of such tumours.

Calcium channel blocker drugs are in clinical use to treat conditions like high blood pressure, angina and tachycardia. In a similar manner, blockers of TRPC calcium channels could be used to inhibit the entry of calcium in tumour cells, thus abrogating cellular proliferation, migration and invasion. A challenge also here is the ubiquitous expression of the TRPC channels in many tissues.

Taken together, the data presented in this thesis is clinically relevant and indicates a need for developing new drugs, thus providing a better prognosis for patients with aggressive and malignant cancers.

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From my desk

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Muhammad Yasir Asghar

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