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The Role of Thyroid Hormones in Graves' Orbitopathy

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CHAPTER 1: INTRODUCTION

1.1. Graves' Disease (GD) and Graves' orbitopathy (GO)

Graves' disease (GD) is an autoimmune hyperthyroid disorder defined by the production of TSHR stimulating autoantibodies (TSAbs), formerly known as long acting thyroid stimulator (LATS), to the thyroid stimulating hormone receptor (TSHR) (Smith and Hall, 1974).

Graves' orbitopathy (GO), also known as thyroid eye disease, generally occurs in hyperthyroid patients or people with a history of hyperthyroidism due to GD (Bahn, 2010). Orbital fibroblasts (OFs), the main target of GO, are known to express TSHR (Bell et al., 2000, Heufelder et al., 1993, Paschke et al., 1994, Stadlmayr et al., 1997, Wakelkamp et al., 2003). Furthermore, TSHR present in GO OFs are responsive to TSHR antibodies (Spitzweg et al., 1997). The strong association between TSHR autoantibodies level and clinical activity score (CAS) of GO together with the presence of TSHR in OFs indicate TSHR as a shared autoantigen between GD and GO (Eckstein et al., 2006, Gerding et al., 2000).

1.1.1. Clinical Features of GD and GO

GD is characterized by an enlarged and overactive thyroid gland (goiter), ocular abnormalities, an accelerated heart rate, and localized dermopathy. More than 50% of GD patients experience weight loss, fatigue, heat intolerance, tremor, and palpitations. Older patients may suffer from atrial fibrillation due to hyperthyroidism (Smith and Hegedus, 2016). GD is the most common cause of hyperthyroidism in iodine-sufficient populations (Taylor et al., 2018).

Approximately 50% of GD patients experience GO symptoms, such as dry and gritty ocular sensation, photophobia, excessive tearing, double vision, and pressure feeling behind the eyes (Bahn, 2010). The common clinical features of GO include upper eye retraction, oedema, erythema of the periorbital tissues and conjunctivae, and proptosis. Among GO patients, around 3-5% experience severe symptoms, such as intense pain, inflammation, and sight-threating corneal ulceration or compressive optic neuropathy (Bahn, 2010).

1.1.2. Epidemiology of GD and GO

The annual incidence of GD is between 20 to 50 cases per 100,000 people. This disease affects approximately 2% of women and 0.2% of men globally. Anyone might carry a risk of developing GD, but adults between the age of 20 and 50 years old are more susceptible to GD (Davies et al., 2020).

In regards to GO, according to a recent report published by the European Group of Graves' Orbitopathy (EUGOGO), the incidence of moderate-to-severe GO in European population was 0.161/10,000/year with a median age of onset of 50 years old. Concerning the sight-debilitating effects caused by GO and the number of incidences which are below than 5/10,000/year, GO is categorized as a rare disease (Perros et al., 2017). Similar to GD, GO is also more common in women. However, men with GO show more severe ocular involvement than women. The age onset of GO is different between women and men. In women, the peak of disease onset occurs between 40-44 years old and between 60-64 years old. Whereas in men, the peak of disease onset occurs between 45-49 years old and between 65-69 years old. The severity of eye disease is higher in older patients (Wiersinga and Bartalena, 2002).

1.1.3. GD Risk Factors

Hereditary, genetic and environmental factors contribute to GD onset in susceptible individuals (Tomer and Huber, 2009). Identical twins have a higher concordance rate of developing GD compared to non-identical twins. Furthermore, family members of GD patients carry a high sibling recurrence risk in developing GD (Brix et al., 2001).

Various genes are known to play a role in GD pathogenesis. These genes can be categorized as either thyroid specific or immune-modulating. The thyroid specific susceptibility genes include thyroglobulin (*Tg*) and *TSHR*. Whereas the immune-modulating susceptibility genes include fork-head box P3 (*FOXP3*), *CD25*, *CD40*, cytotoxic T-lymphocyte associated factor 4 (*CTLA-4*), and human leukocyte antigen (*HLA*) (Lee et al., 2015).

TSHR is the major thyroid specific gene associated with GD (Dechairo et al., 2005). Not only in the thyroid and on OFs, functional TSHR is also expressed in thymocytes which are precursors of T cells (Giménez-Barcons et al., 2015). In addition to the full length TSHR, there are five truncated TSHR isoforms generated by alternative splicing. All of

these TSHR isoforms lack of a transmembrane-spanning region. Among those TSHR isoforms, ST4 and ST5 are the most prominent (Hunt et al., 1995).

One of the most important genetic factors that increase the risk of developing GD in susceptible individuals is single nucleotide polymorphisms (SNPs) located in intron 1 of *TSHR* (Brand et al., 2009). A subsequent study proposed that SNPs in intron 1 of *TSHR* gene correlates with allele-specific intra-thymic expression level of TSHR (Colobran et al., 2011). Furthermore, in a more recent study it was demonstrated that SNPs in intron 1 of TSHR induces an unbalanced transcription between protective and predisposing allele in the thymus. The protective TSHR allele was preferentially transcribed (Marín-Sánchez et al., 2019). The authors also found high levels of short TSHR transcripts expression, especially ST4, in the thymus. Finally, they proposed that failure of TSHR tolerance in GD is caused by lack of presentation of full-length TSHR molecule by tolerogenic APCs in thymic medulla (Marín-Sánchez et al., 2019). Recently, an *in silico* modelling study reported that a TSHR truncated isoform called TSHRv1.3 (Graves et al., 1992) (another term for ST4) has TSH and autoantibody binding ability (Latif et al., 2019). The authors reported that TSHRv1.3 release into the circulation as a functional soluble receptor may modulate GD pathogenesis (Latif et al., 2019).

Another genetic risk factor of GD is sex (gender). Brix and colleagues reported the association of skewed X-chromosome inactivation in female blood with GD occurrence. The authors proposed that this genetic pattern partly explains the high prevalence of GD in female (Brix et al., 2005).

While genetic factors highly contribute to GD onset, around 20% of GD risk factors are non-genetic or come from the environment. Among these non-genetic factors are viral infection, stress, and iodine-containing drugs (Davies et al., 2020). Viral infection is known to trigger the genetic-epigenetic mechanism in GD (Stefan et al., 2014). Local cytokine production, such as interferon (IFN) α, is known to mediate viral-induced autoimmunity (Akeno et al., 2011). The presence of IFNα-induced histone methylation was found in SNPs in intron 1 of TSHR, which is associated with GD. On the other hand, promyelocytic leukemia zinc finger protein (PLZF), an IFNα-mediated transcription factor, was also expressed in thyroid and thymus cells. PLZF acts as a TSHR repressor. However, the presence of PLZF in the thymus led to downregulation of TSHR protective allele, instead of TSHR predisposing allele due to the low expression of the latter. This

mechanism was reported to contribute on the failure of central tolerance to TSHR (Stefan et al., 2014).

Stress is also often associated with the onset of GD, especially in susceptible individuals. Sharif and colleagues, in their recent review, reported that most GD patients had suffered from more than one stressful events in their life less than 12 months prior to GD diagnosis. In addition, there is a higher change to develop relapse in GD patients who experience hypochondriasis, depression, and paranoia (Sharif et al., 2018). Iodine-containing drug such as amiodarone, which is commonly used to treat ventricular fibrillation and tachycardia, can also trigger GD development in susceptible individuals as well as in individuals without underlying thyroid diseases (Basaria and Cooper, 2005).

1.1.4. Thyroid stimulating hormone receptor (TSHR)

TSHR is the major autoantigen in GD (Smith and Hall, 1974). Human TSHR was first cloned from human thyroid cDNA library in 1989 (Libert et al., 1989, Misrahi et al., 1990, Nagayama et al., 1989, Nagayama and Rapoport, 1992). TSHR is evolutionary close relative to follicle-stimulating hormone receptor (FSHR) and lutropin choriogonadotropin receptor (LCHGR). Together, they constitute the sub-family of glycoprotein hormone receptors (GPHRs) (Vassart et al., 2004). TSHR is a member of the class A G-protein coupled receptors (GPCRs) (Fredriksson et al., 2003). It is important for thyroid growth and function (Postiglione et al., 2002), activation of different G-protein subtypes (Laugwitz et al., 1996), and signalling pathways (Krause et al., 2012). Importantly, TSHR is also expressed in extra-thyroidal tissues, such as orbital adipose tissues (Bahn et al., 1998), pre-adipocytes (Haraguchi et al., 1996), kidney (Sellitti et al., 2000), brain (Crisanti et al., 2001), and bone (Inoue et al., 1998).

Currently, TSHR structure is known to consist of three parts which are the leucine rich repeat domain (LRRD), the hinge region, and the membrane spanning serpentine domain (SD) (Kleinau et al., 2017), as shown schematically in Figure 1. The extracellular LRRD and hinge region occupy the N-terminal extracellular receptor part (Kleinau et al., 2011). Thyroid stimulating hormone (TSH) and TSHR autoantibodies bind TSHR in this region (Chen et al., 2010, Sanders et al., 2007, Sanders et al., 2011). The LRRD has a scythe blade-like shape with a slight twist from the N- towards C-terminus. According to the solved FSHR LRRD structure, TSHR LRRD was predicted to consist of 12 repeats (Jiang

et al., 2012). Two cysteines located at the last C-terminal repeats of LRRD interact with two cysteines at the C-terminal hinge region and form disulphide bridges. These disulphide bridges connect LRRD to the membrane-spanning serpentine domain (SD) (Jiang et al., 2012). Currently, the structure of TSHR hinge region is not entirely known. TSHR is known to be enzymatically cleaved at two sites in the hinge region, which is a prerequisite for shedding of the disulphide bridges (Chazenbalk et al., 1997). The combination of shedding and cleavage generates two TSHR subunits, A and B. TSHR subunit A consists of LRRD and parts of hinge region, while TSHR subunit B consists of C-terminal part of N-terminus and the membrane-spanning SD (Chazenbalk et al., 1997). The solved FSHR ectodomain crystal structure revealed that hinge region is an integral part of the ectodomain (Jiang et al., 2012). Moreover, this crystal structure bound with FSH showed the details of the second hormone-binding site of GPHRs between the receptor subunits, which strongly contributes to hormone-binding properties (Jiang et al., 2012).

There is currently no structural information on TSHR SD, which comprises the seven membrane-spanning helices and respective connecting loops. However, with the assumption that TSHR generally shares a common structural organization with other class A GPCRs, experimental models have been developed to elucidate mechanism of pathogenic mutations (Ringkananont et al., 2006), allosteric small-molecule binding (Latif et al., 2016), or G-protein and arrestin coupling (Kang et al., 2015).

Induction of endogenous signalling cascades is triggered extracellularly by TSH (Tate et al., 1975). As mentioned before, LRRD and hinge region of TSHR have ligand-binding sites (Jiang et al., 2012). Furthermore, the hinge region consists of a tethered ligand that mediates extracellular signal transduction (Majumdar and Dighe, 2012). There is an interaction between the hinge region and extracellular loops which connect SD helices. That interaction transmits the signal toward the transmembrane region (Kleinau et al., 2008).

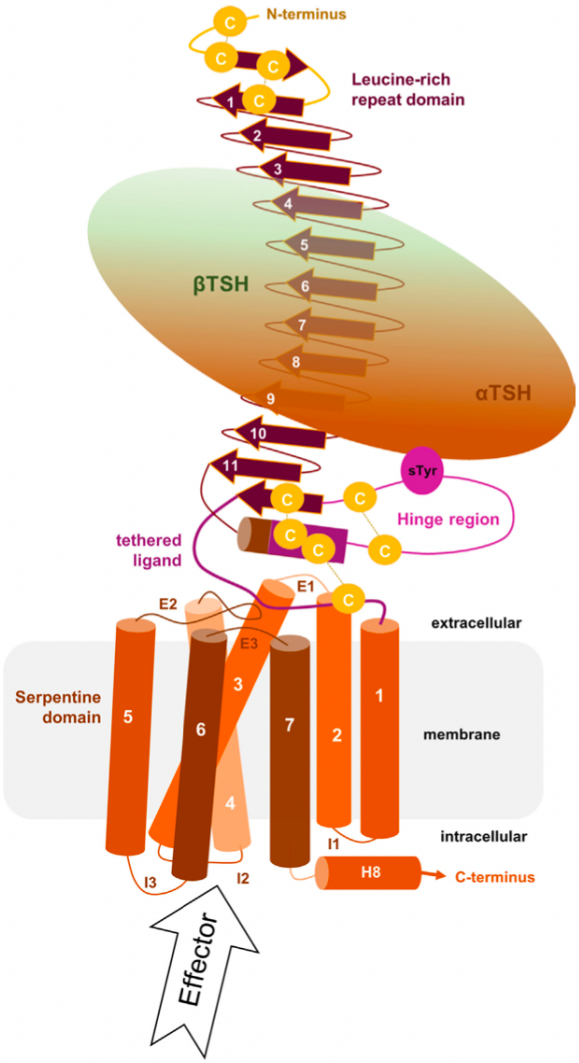

Figure 1. Scheme of the overall TSHR protein structure. The LRRD together with the hinge region constituting the extracellular part of TSHR. The seven transmembrane helices and their connecting loops arrange the SD, which spans the membrane from the extra-to the intracellular part. A tethered ligand located between the extracellular loops is composed of amino acids from C-terminal ends of the LRRD and the hinge region (Kleinau et al., 2017).

1.1.5. TSHR signalling

TSH binding to TSHR activates both cyclic adenosine monophosphate (cAMP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) cascades (Van Sande et al., 1990). These cascades are mediated by G_s and $G_{q/11}$ pathways, respectively (Allgeier et al., 1994). Most of TSHR activities are mediated by G_s protein (Kleinau and Biebermann, 2014, Latif et

al., 2009, Vassart and Dumont, 1992). G_q/G_{11}-mediated signalling pathway is important for TSH-induced TH synthesis and release (Kero et al., 2007).

Kleinau and colleagues generated the first model of TSHR/G-protein interaction based on the crystal structure of opsin on complex with the C-terminal helical peptide of transducin (Kleinau et al., 2010). This model shows that the contact between TSHR and G_q occurs at intracellular loop 1 and transmembrane helix 8 of TSHR transmembrane region. Furthermore, this model illustrates that the binding mode between TSHR/G_s and TSHR/G_q is partially overlap (Kleinau et al., 2010).

A study on rhodopsin revealed that G-protein-mediated signalling can also be attenuated and desensitized by recruitment of arrestins (Krupnick et al., 1997). Light-activated rhodopsin is phosphorylated by GPCR kinases (GRKs). Subsequently, arrestins bind the intracellular loops and/or C-terminus of the phosphorylated receptor and terminate G protein signalling (Krupnick et al., 1997). The detailed interaction between TSHR and arrestins was further studied using crystal structure of rhodopsin (Kang et al., 2015). This model showed that arrestins bound to the transmembrane helix 7 and 8 of the GPCRs (Kang et al., 2015).

1.1.6. TSHR autoantibodies

The autoimmune mechanism in GD is mainly triggered by TSAbs. There are three types of TSHR autoantibodies, namely stimulating, blocking, and neutral autoantibodies (Morshed and Davies, 2015). TSAbs stimulate TSHR activities by competing with TSH for receptor binding site (Smith and Hall, 1974). The generation of M22, monoclonal antibody (mAb) that display TH-stimulating activities, has made studies on TSAbs characteristics and functions possible. This mAb inhibits TSH binding to TSHR and stimulates cAMP production in Chinese hamster ovary cells that express TSHR (Sanders et al., 2003, Sanders et al., 2004). According to the analysis on the crystal structure of M22-TSHR complex, M22 was found to bind the concave surface of TSHR LRRD, which expands from the N terminus to the C terminus (Sanders et al., 2007). M22 uses similar signalling pathway as TSH, which are G_s and G_q. However, unlike TSH, M22 mAb stimulation was reported to significantly increase cAMP/protein kinase A (PKA)/cAMP-responsive element binding (CREB) activity as well as Akt and protein kinase C (PKC) phosphorylation (Morshed et al., 2009).

TSHR blocking autoantibodies (TBAbs) were first detected in immunoglobulin G (IgG) preparation from patients with autoimmune hypothyroidism (Chiovato et al., 1987). These autoantibodies act as antagonists and prevent TSH binding to TSHR. Furthermore, TBAbs inhibit TSH- induced cAMP production (Chiovato et al., 1987). The human TSHR blocking mAb, K1-70, was isolated from the lymphocytes of hypothyroid patients (Evans et al., 2010). Analysis performed on the K1-70 and TSHR complex crystal structure revealed that K1-70 binds TSHR LRRD in a similar way as M22, but is in approximately the opposite orientation. K1-70 was found to bind more N-terminal on the TSHR LRRD and interacts with a large surface of the receptor, while M22 and TSH interact more with the C-terminus. This composition reflects K1-70 biological activities, which are different to M22 and TSH (Sanders et al., 2011).

TSHR neutral autoantibodies (TNAbs) do not block TSH binding to TSHR nor induce cAMP via G_s (Tonacchera et al., 1996). The binding site for TNAbs is at the cleaved region of TSHR. Therefore, they were also called cleavage autoantibodies (Ando et al., 2004). The human neutral TSHR mAb is currently unavailable. Studies on TNAbs have been performed using hamster and mouse neutral TSHR mAbs (Morshed et.al., 2009, Morshed et.al., 2010). Two different neutral mAbs have been reported to exert different mechanism in rat thyroid cells. One neutral mAb suppressed multiple signalling cascades, while the other activated them (Morshed et al., 2009). According to a study conducted on rat thyroid cells, TNAbs could activate the non-classical TSHR signalling, such as protein kinase C (PKC)/mitogen activated protein kinase (MAPK), mechanistic target of rapamycin (mTOR)/S6K, nuclear factor kappa-b (NF-κB), certain cytokines, oxidative stress signalling, and induce apoptosis (Morshed et al., 2010). Recently, it was reported that MC1, one of the neutral TSHR mAbs, induced mild hypothyroidism in BALB/c mice. Furthermore, MC1 induced the formation of reactive oxygen species (ROS) in thyroid cells and severe stress in the cellular organelles. These led to thyroid cell death via apoptosis (Morshed et al., 2019).

1.1.7. GD pathogenesis

GD pathogenesis is initiated by thyroid-infiltrating autoreactive B cells and T cells that escape central tolerance. It is known that $CD4^+$ T cells (helper T cells) are more abundant than $CD8^+$ T cells (effector T cells) in thyroid cells (Margolick et al., 1988). The

infiltrating $CD4^+$ T cells secrete interferons, which induce the expression of major histocompatibility complex (MHC) class II in GD thyroid tissues (Piccinini et al., 1988). Furthermore, autoreactive $CD4^+$ T cells receptors interact with major MHC class II molecules, through which TSHR peptides are presented to T cells which lead to T cells activation (Martin et al., 1989). Infiltrating autoreactive B cells differentiate into autoantibody-producing plasma cells through the interaction between CD40 expressed on the B-cell surface and CD40 ligand expressed by $CD4^+$ T cells (Faure et al., 1997). B cell population isolated from the blood of GD patients was reported to display insulin-like growth factor 1 receptor (IGF1-R) expression which enhance TSHR autoantibodies production (Douglas et al., 2008). All of these mechanisms lead to thyroid inflammation and TH (3,5,3'-triiodothyronine and 3,5,3',5'-tetraiodothyroxine) overproduction by thyroid cells which are the hallmarks of GD (Davies et al., 2020).

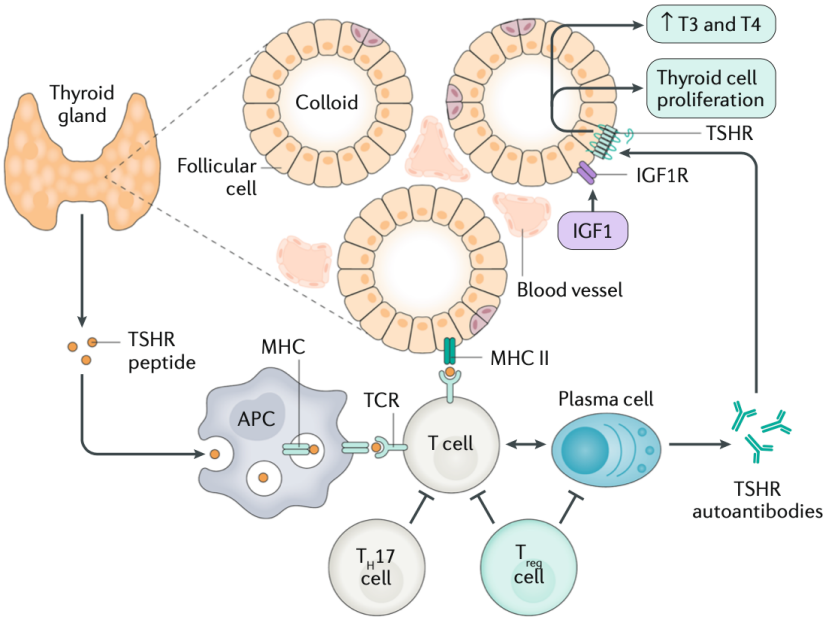

Figure 2. Graves' disease pathogenesis. Autoreactive T cells and B cells escaped central tolerance and are activated by TSHR peptides presented by antigen presenting cells which might be the thyroid cells themselves, macrophages, B cells, or dendritic cells. TSAbs are produced by autoreactive B cells and enhanced by IGF-1R. All of these mechanisms stimulate thyroid cells to produce TH, resulting in clinical manifestations of hyperthyroidism (Davies et al., 2020).

1.1.8. GO risk factors

While the risk factors of GD also apply in GO, there are additional risk factors of GO such as high TSAbs titres, smoking, radioiodine treatment, and gut microbiota (Davies et al., 2020). High TSAbs level has been associated with higher risk of GO development in GD patients (Khoo et al., 1999). In addition, patients with severe GO showed higher TSAbs level, compared to patients with mild GO and the high level of TSAbs persist during the course of GO (Eckstein et al., 2006). Smoking increased the risk of more severe GO (Prummel and Wiersinga, 1993). Furthermore, GO patients who are smokers showed delayed and poor response towards GO therapies (Eckstein et al., 2003). Most of GD patients with severe hyperthyroidism who undergo radioiodine therapy were reported to show worse autoimmune reaction towards TSHR, compared to GD patients who are treated with other types of treatment. This was characterized by TSAbs upregulation following radioiodine treatment, which leads to a higher risk of developing GO (Laurberg et al., 2008). Recent studies on GO animal models revealed the involvement of gut microbiota in influencing GO outcome (Masetti et al., 2018, Moshkelgosha et al., 2018). Those animal studies were confirmed in a clinical study that describe alterations in gut microbiota population as a GO risk factor (Shi et al., 2019).

1.1.9. GO pathogenesis

During the active phase of GO, orbital tissues are infiltrated with lymphocytes which mainly consist of T lymphocytes and macrophages. There are only a few B cells and natural killer (NK) cells in the infiltrate (Weetman et al., 1989). Both $CD4^+$ and $CD8^+$ T cells were found to infiltrate GO orbital tissues (Förster et al., 1998). In addition, MHC Class II molecules and interferons were also detected in the orbital tissues (HEUFELDER et al., 1991).

OFs are known to be the target cells in GO (Bahn et al., 1987). Interactions between T cells and OFs results in orbital tissue activation and induction of gene expression which involved in inflammation and tissue remodelling (Bahn et al., 1987). TSHR, known as GD autoantigen, presents in GO OFs as well as control OFs (Heufelder et al., 1993). Furthermore, active GO patients show a much higher TSHR expression compared to inactive GO patients (Wakelkamp et al., 2003). The positive correlation between TSHR

autoantibody titres and GO severity in GD patients indicates TSHR as the shared autoantigen between GD and GO (Eckstein et al., 2006).

Upon TSHR exposure, infiltrating T cells differentiate into Th1, Th2, Th17, and regulatory T cells (Tregs). A study on orbital fat tissues derived from active GO patients showed a predominant expression of Th1-like cytokines. That study also reported the similar expression of Th2-like cytokines in active and inactive GO (Wakelkamp et al., 2003). Th17 expression positively correlates with disease activity of the GO patients (Fang et al., 2016). Th17 cells enhance pro-inflammatory cytokine production by OFs. In addition, Th17 cells upregulate costimulatory molecules in $CD34^+$ OFs population. On the other hand, OFs produce prostaglandin E2 (PGE_2) to support Th17 cells differentiation (Fang et al., 2016). It was recently reported that there is a T-cell immunity shift from Th1-bias to Th-17 bias in GD patients who develop GO. IFN-γ producing Th1, IL-17A-producing Th17.1, and memory Th1 cells are the independent factors associated with GO development in GD patients. In addition, IFN-γ producing Th17.1 is the independent marker of very severe GO development (Fang et al., 2020). Low level of Tregs was also detected in peripheral blood mononuclear cells (PBMC) of GO patients (Kahaly et al., 2011).

GO OFs can be divided into two populations based on the presence of CD90. OFs displaying CD90 ($CD90^+$) produce hyaluronan and PGE_2, IL-6, and are capable of myofibroblast differentiation. Whereas, OFs lacking CD90 ($CD90^-$) differentiate into mature adipocytes, express MHC Class II surface receptor (HLA-DR), and produce high level of IL-8 following activation (Koumas et al., 2003, Koumas et al., 2002). GO OFs were further reported to consist of $CD34^+$ bone marrow-derived fibrocytes that also express TSHR and had a potency to differentiate into adipocytes (Douglas et al., 2010). OFs derived from GO patients show elevated CD90 expression (Khoo et al., 2008). GO OFs was also demonstrated to express CD40, a co-stimulatory molecule which is upregulated following IFN-γ stimulation (Sempowski et al., 1998). CD40 and CD154 (CD40 ligand) ligation together with MHC Class II-T cell receptor (TCR) complex are involved in physical interactions between GO OFs and T-lymphocytes (Feldon et al., 2005). Recently, CD40 stimulation with CD40 ligand has been identified to induce sphingosine-1-phosphate (S1P) and attract T cell migration to GO OFs (Plöhn et al., 2018). These interactions led to T cell activation, GO OF proliferation, and hyaluronan

production (Feldon et al., 2005). In addition, GO OFs were reported to express high amount of IGF1R (Tsui et al., 2008). The crosstalk between TSHR and IGF1R enhances TSAbs stimulation in GO OFs (Krieger et al., 2016). Furthermore, GO OFs express peroxisome proliferator activated receptor (PPAR)-γ. The interaction between this transcription factor and its ligands expressed by activated T cells drive OF adipogenesis (Feldon et al., 2006).

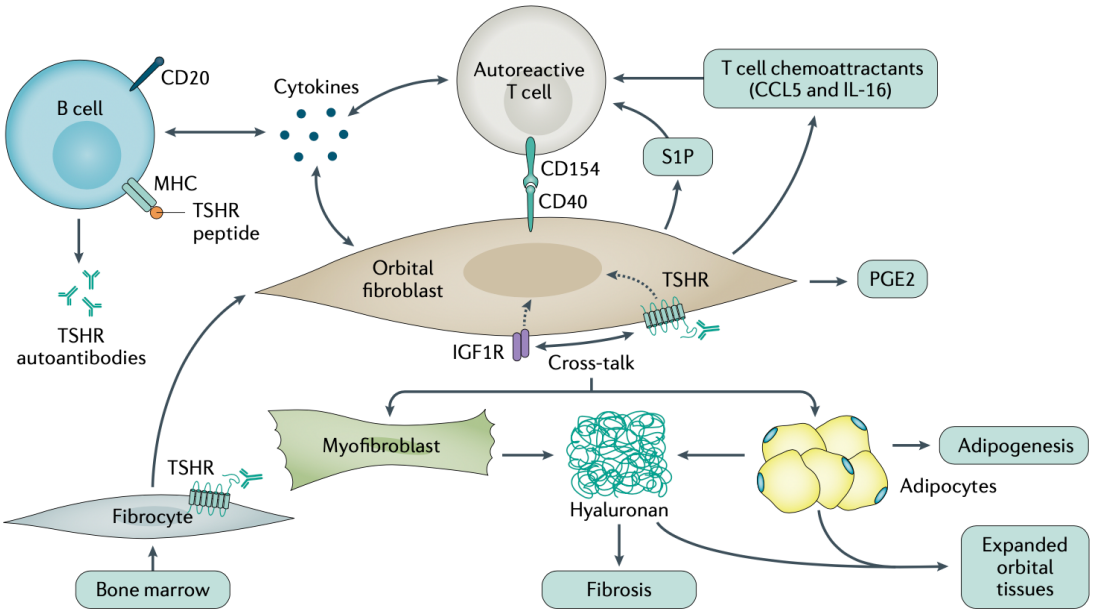

Figure 3. Pathogenesis of Graves' orbitopathy. Orbital fibroblasts (OFs) are the main target of GO. They can differentiate into myofibroblasts and adipocytes. The binding of CD40 expressed by OFs with its ligand on T cells induce T cells migration via S1P. In addition, interaction between MHC and TCR also activate T cells and trigger B cells to produce TSHR autoantibodies. OFs are activated by the interaction between TSHR autoantibodies and TSHR, which induce a cross-talk with IGF1R (Davies et al., 2020).

1.1.10. GD induction in mouse models

Early attempts to induce GD in mouse models were done through immunization with either human or mouse TSHR-expressing cells (Banga, 2007). The first GD mouse model was known as the Shimojo model (Shimojo et al., 1996). This model was established by immunizing mice with fibroblasts transfected with both human TSHR and MHC Class II molecule. While TSAbs and thyrotropin binding inhibiting immunoglobulins (TBII)

displayed by this mouse model and changes in thyroid gland as well as increased TH level were apparent, there was no signs of immune cells infiltration in the thyroid follicular cells (Shimojo et al., 1996). The second model was generated by immunizing BALB/c mice using M-12 (a mouse-derived B lymphoblasts) expressing either mouse or human TSHR. This method led to the detection of lymphocytic infiltration in thyroid cells (Kaithamana et al., 1999). Finally, Kita-Furuyama and colleagues injected DCs infected with adenovirus-expressing TSHR into BALB/c mice, which revealed the Th1-dominant cytokines in splenocytes in response to TSHR antigens (Kita-Furuyama et al., 2003).

The first successful genetic immunization to generate GD in a mouse model was performed by Nagayama and colleagues (Nagayama et al., 2002). In the study, they immunized different strains of mice with adenovirus expressing TSHR or β-Gal. 55% of female and 33% of male BALB/c and 25% of female C57BL/6 mice developed Graves'-like hyperthyroidism with elevated serum T_4 levels and positive TSHR autoantibodies, with thyroid stimulating immunoglobulin (TSI) and TBII activities. Furthermore, they found TSHR epitopes presented in the immunized mice were similar to the one in GD patients. However, this mouse model did not show inflammatory cell infiltration (Nagayama et al., 2002).

The Nagayama model was modified by Chen and colleagues (Chen et al., 2003). This group performed immunization to BALB/c mice with adenovirus that expresses free A subunit of TSHR (termed TSHR A-subunit). This method resulted in higher induction of TSAbs in immunized mice compared to the previous model (Nagayama et al., 2002, Chen et al., 2003). This is currently the most widely used and highly reproducible GD mouse model.

1.1.11. Establishment of GO in GD mouse models

The first successful GO induction in a GD mouse model was performed by immunization using plasmids encoding hTSHR A-subunit or IGF1Rα to female BALB/c via electroporation (Zhao et al., 2011). The combination of plasmid injection and electroporation was first performed by Kaneda and colleagues (Kaneda et al., 2007). This approach led to TSAbs production and hyperthyroidism in BALB/c mice that lasted for more than 8 months after immunization (Kaneda et al., 2007). In their study, Zhao and colleagues reported that the mice immunized with hTSHR A-subunit plasmid showed

high frequency of hyperthyroidism and TSAbs production. Furthermore, these mice produced antibody against the IGF1Rα subunit. However, despite showing response to IGF1R, mice immunized with IGF1Rα plasmid did not show any phenotypic changes. Histological analysis of orbital tissues showed a moderate connective tissue fibrosis, suggesting that this is the first mouse model demonstrating a close association between GD and GO (Zhao et al., 2011).

This mouse model was developed further by Moshkelgosha and colleagues (Moshkelgosha et al., 2013). This model displayed more features of GO compared to the previous model developed by Zhao and colleagues, such as T cells and immune cells infiltration to the orbital muscle tissue. However, most of the hTSHR A-subunit plasmid immunized mice were hypothyroid (Moshkelgosha et al., 2013). A comparative study conducted on this mouse model in two laboratories showed similar GO following immunization with hTSHR-A subunit plasmid features. Nevertheless, the TH status was inconsistent (Berchner-Pfannschmidt et al., 2016). The gut microbiota composition in mice established in different environment was later found to influence difference in TH status (Masetti et al., 2018). The pathological features of GO have been successfully replicated in other studies using this mouse model (Plohn et al., 2019, Schluter et al., 2018). Therefore, hTSHR-A subunit plasmid electroporation is presently the most effective method to generate a robust mouse model to study GO pathogenesis.

Another existing GO mouse model was developed through a long term immunization to female BALB/c mice with recombinant adenovirus expressing hTSHR A-subunit (Holthoff et al., 2017). This model consistently demonstrated pathological GD phenotypes accompanied by retro-orbital fibrosis over 9 months (Holthoff et al., 2017) and has been recently replicated by a Chinese group (Zhang et al., 2021)

1.1.12. Immunomodulatory studies in GD/GO mouse models

The immunomodulatory studies have been widely performed in adenovirus carrying TSHR subunit A-immunized mouse model. Treatment using cyclic peptide that mimic one of the cylindrical loops of TSHR LRRD was reported to stabilize TSAbs titres despite continuing immunizations, reduce thyroid hyperplasia, normalize T_4 levels, and minimize orbital fibrosis (Holthoff et al., 2017). Furthermore, the same group reported that the administration of 11-mer cyclic peptide (P19) derived from the first loop of TSHR LRRD

to Ad-TSHR289-immunized mice resulted in high affinity inhibition of anti-TSHR antibody (Faßbender et al., 2019). This study was recently replicated in another laboratory and gave similar results, suggesting that P19 might be a potential therapy for GD and associated GO (Diana et al., 2021).

Another study confirmed the role of 5α-dihydrotestosterone (DHT) in reducing GD severity in female BALB/c mice by regulating oxidative stress and immunosuppressive cytokines (Zhao et al., 2019). A recent study by our group described the role of fingolimod, an S1P receptor modulator, in improving disease outcome in GD/GO mouse model (Plohn et al., 2019). Fingolimod administration before disease onset prevented TSAb formation in TSHR-immunized mice. Furthermore, fingolimod treatment after disease onset was successfully suppressed GD/GO in half of the animals, while reduced the disease progression in the remaining half of the animals (Plohn et al., 2019).

A study on a HLA-DR3 transgenic mouse model showed that the administration of TSHR-derived antigen-processing independent epitopes (apitopes) could induce tolerance toward TSHR autoantibodies (Jansson et al., 2018). This study has been translated into a phase I clinical trial (Pearce et al., 2019). The administration of two TSHR peptides, termed ATX-GD-59, into untreated mild to moderate GD patients resulted in improved serum free T_3 and T_4 level in 7 out of 10 patients. These changes in free TH serum level were positively correlated with reduced concentration of serum TSHR autoantibody (Pearce et al., 2019). Therefore, ATX-GD-59 was predicted to be a safe and well-tolerated treatment for GD (Pearce et al., 2019).

1.2. Thyroid hormones (TH)

TH have a prominent role in regulating normal function of almost all tissues in the human body, such as maintaining growth (Yaffe and Samuels, 1984) and metabolic rate (Mariash et al., 1980).TH are produced by thyroid gland, as T_4 and T_3 (Gross and Pitt-Rivers, 1951). While T_4 is the majority of TH secreted by thyroid gland to the circulation, T_3 has been considered as the most active form because it binds nuclear receptors with much higher affinity compared to T_4 (Oppenheimer et al., 1974).

1.2.1. TH synthesis and release

TH synthesis and release are regulated by the hypothalamic-pituitary-thyroid (HPT) axis. The paraventricular nucleus (PVN) of the hypothalamus secrete thyrotropin releasing hormone (TRH). TRH stimulates anterior pituitary to produce TSH (Menezes-Ferreira et al., 1986). In addition, TSH production is under a negative feedback control of TH (Shupnik et al., 1985). TSH stimulates the activation of sodium/iodide symporter (NIS) located in the basolateral membrane of thyroid gland epithelial cells (Weiss et al., 1984). NIS takes up iodide that reach thyroid cells through the bloodstream (Bagchi and Fawcett, 1973) and involved in TH synthesis.

The first step of TH synthesis is iodide oxidation and attachment of iodine to tyrosyl residue within the thyroglobulin (Tg). The iodination of tyrosyl residues in Tg results in the formation of mono-iodinated (MIT) and di-iodinated (DIT) ring. MIT and DIT are then transferred to another DIT tyrosine, making the immediate precursors of T_3 and T_4 (Gavaret et al., 1981). These steps are catalysed by thyroid peroxidase (TPO) (Virion et al., 1979). Some studies demonstrated that Tg performs auto-regulatory mechanism in relation to TH synthesis (Ishido et al., 2014, Suzuki et al., 1999a, Suzuki et al., 1999b). An initial study conducted on rat thyroid FRTL-5 cells reported that treatment with Tg at physiological concentrations suppressed gene expression of key molecules in TH synthesis, including *Tg* gene itself (Suzuki et al., 1999a). Two other studies confirmed the negative-feedback effect of follicular Tg in rat thyroids *in vivo* (Suzuki et al., 1999b) and in primary cultures of normal human thyrocytes (Ishido et al., 2014). This negative feedback mechanism was later described to be mediated by two lipid raft proteins, flotilin 1 and flotilin 2 (Luo et al., 2016).

Finally, Tg is transported by coated vesicles from the thyroid follicular cells to lysosomal compartment via endocytosis (Bernier-Valentin et al., 1990). Lee and colleagues proposed that this intracellular transport of Tg is dependent on intramolecular chaperone and escort function of its cholinesterase-like domain (Lee et al., 2008). Under the regulation of TSH, thyroid follicular cells secrete specific proteolytic enzymes called Cathepsin B. These enzymes are transported to the plasma membrane and are secreted into the extracellular space, where they catalyse Tg degradation and induce T_3 and T_4 release (Linke et al., 2002).

Following TH release, 99% of TH bind TH distributor proteins, such as thyroxine-binding globulin (TBG), transthyretin (TTR), and albumin that mediate their transport to the circulation. The function of these TH distributor proteins is to ensure uniform distribution of TH within tissues throughout the body by maintaining the quantity of free TH in the circulation (MENDEL et al., 1987).

1.2.2. TH transport

TH transport across the cell membrane is required for their actions in the target cells. TH transport performed through transmembrane TH transporters (Groeneweg et al., 2020). Currently there are five different TH transporter protein families, namely organic anion transporters (OATPs, SLC10, and SLC17), L-type amino acid transporters (LATs), and monocarboxylate transporter (MCTs, also known as SLC16). Currently, the most efficient TH transporters are MCT8, MCT10, OATP1C1, and SLC17A4 (Groeneweg et al., 2020).

Similar to human MCT8, human MCT10 facilitates bidirectional transport of T_3 and T_4 across the plasma membrane (Friesema et al., 2008). OATP1C1 mediates transport of T_4, T_4 sulfate, and rT_3 (van der Deure et al., 2008). This protein is important for delivering serum TH to the brain. Furthermore, OATP1C1 plays an important role in regulating T_4 concentration in the central nervous system and in brain development (Sugiyama et al., 2003). A recent genome wide analysis study reported that *SLC17A4* encodes a high-affinity T_3 and T_4 transporter (Teumer et al., 2018). This gene is predominantly expressed in human small intestinal and colonic epithelial cells, pancreas, liver, and kidney cortex (Togawa et al., 2012). Therefore, SLC17A4 has been proposed to contribute in metabolic clearance and entero-hepatic cycle of TH (Teumer et al., 2018).

Monocarboxylate transporter 8 (MCT8)

Among all known TH transporters, MCT8 is the most specific TH transporter (Friesema et al., 2003). Rat MCT8 facilitates cellular uptake of T_4, T_3, and 3,3'-T_2 as well as the inactive metabolite rT_3. While T_4 transport by MCT8 is inhibited in the absence of Na^+, T_3 transport is Na^+-independent. MCT8 is highly expressed in liver, kidney, brain, heart (Friesema et al., 2003), and thyroid gland (Di Cosmo et al., 2010).

Human *MCT8* gene consists of 6 exons, encoding a protein with 12 putative transmembrane domains. This gene is located in the X chromosome (Lafrenière et al., 1994). MCT8 is the only MCT member that contains N-terminal PEST domain (a domain with abundant proline, glutamate, serine, and threonine amino acids) which make this protein prone to degradation (Lafrenière et al., 1994). Human MCT8 facilitates the uptake of T_4 and T_3 but not 3,3'-T_2, and rT_3 (Friesema et al., 2006). Not only facilitating TH uptake, human MCT8 together with MCT10 also facilitate TH efflux (Friesema et al., 2008).

MCT8 is present at the basolateral membrane of thyrocytes and involved in TH secretion from the thyroid gland (Di Cosmo et al., 2010, Trajkovic-Arsic et al., 2010) as well as maintaining thyroid autoregulation (Venugopalan et al., 2021, Weber et al., 2017). MCT8 interacts with TSHR in the thyrocytes. The TSHR/MCT8 complex modulate the signalling capacity and pathway selectivity of TSHR (Fischer et al., 2018). GD patients show a high level of MCT8, which is consistent to increased TH release (Badziong et al., 2017). Furthermore, as reported by Fischer and colleagues, TSHR expression is lower than MCT8 in GD patients (Fischer et al., 2018).

1.2.3. TH activation and inactivation by deiodinases

Deiodination is the first step of TH action in the target cells. This process is mediated by deiodinase enzymes, deiodinase 1 (DIO1) and deiodinase 2 (DIO2), which convert T_4 into T_3. DIO1 and DIO2 were initially distinguished based on their sensitivity towards 6-*n*-propyl-2 thiouracyl (PTU) inhibition (Oppenheimer et al., 1972, Silva and Larsen, 1977). DIO1 is PTU-sensitive (Oppenheimer et al., 1972), while DIO2 is PTU-resistant (Silva and Larsen, 1977).

DIO1 mediates the conversion of T_4 to T_3. The function of DIO1 is to limit the increase of serum T_3 under hyperthyroid condition (Schneider et al., 2006). Most of the T_3 generated from T_4 in normal human thyroid is catalysed by DIO1 (Ito et al., 2011). In rat thyroid cells, DIO1 expression was shown to be stimulated by TSH (Borges et al., 1990).

DIO2 catalyses the conversion of T_4 to T_3 and rT_3 to 3,3'-T_2. This enzyme is important for the feedback regulation of TSH secretion by circulating T_4 (Schneider et al., 2001). DIO2 is known to produce most of the T_3 required by human body each day (Maia et al., 2005). While not being expressed in rat thyroid cells (Borges et al., 1990), *DIO2* mRNA

is expressed in human thyroid and is upregulated in the thyroid derived from GD patients. Therefore, in GD patients, T_3 can be produced via DIO2-mediated deiodination of T_4 as well as direct hydrolysis of Tg (Ito et al., 2011). DIO2 expression in brown adipose tissue (BAT) is highly induced by cold exposure (Silva and Larsen, 1983) and during differentiation.

Deiodinase 3 (DIO3) catalyses the conversion of T_4 to rT_3 and of T_3 to 3,3'-T_2 which are biologically inactive. DIO3 maintains TH homeostasis by protecting tissues from excess TH (Escobar-Morreale et al., 1997). DIO3 activity and mRNA are strongly induced during BAT proliferation *in vitro* but decreased during differentiation (Hernandez et al., 2007). The presence of DIO3 has been speculated to induce oxidative stress in GO orbital adipose tissue (Van Regemorter et al., 2021). Van Regemorter and colleagues recently reported DIO3 upregulation in the orbital adipose tissue derived from GO patients, which led into T_3 depletion in the cytoplasm of GO orbital adipocytes (Van Regemorter et al., 2021).

1.2.4. TH receptors

TH actions are mainly mediated by thyroid hormone receptors (THR) (Sap et al., 1986, Weinberger et al., 1986). THR modulates target genes transcription in response to T_3. THR normally binds thyroid response element (TRE) at the responsive genes as a heterodimer with retinoid x receptor (RXR) (Shibusawa et al., 2003). In the absence of ligand (T_3), THR binds positive TRE via two zinc fingers at the DNA binding domain (DBD) and repress gene expression together with the co-repressors. Upon T_3 binding, THR undergoes conformational change, release co-repressors and recruit coactivators. Additionally, THR can repress gene expression in a T_3-dependent manner by binding to negative TRE (Shibusawa et al., 2003).

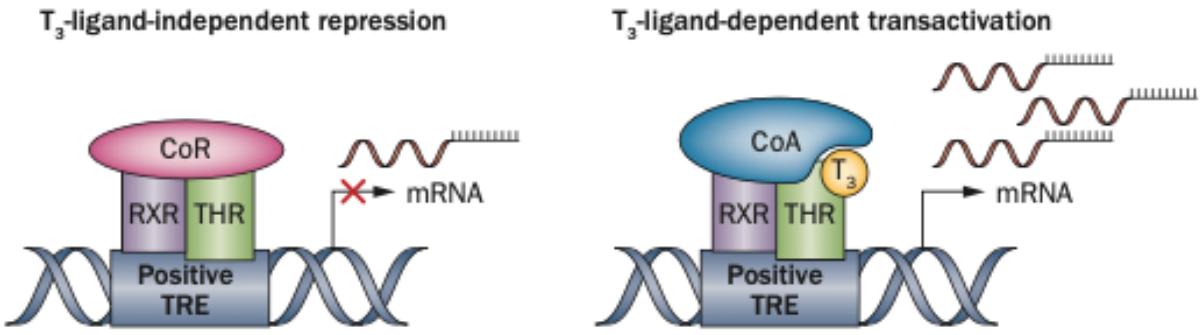

Figure 4. Model of TH responsive gene regulation. In the absence of T_3, a co-repressor is bound to RXR-THR heterodimer at the positive TRE. This mechanism represses target gene expression. In the presence of T_3, the co-repressor is released and co-activators are recruited. This results in gene transcription activation (Ortiga-Carvalho et al., 2014).

There are three major THR isoforms, THRA1, THRB1 and THRB2 that are the members of nuclear receptor family (Hodin et al., 1989, Koenig et al., 1988, Sap et al., 1986). THR isoforms are encoded by two genes, *THRA* and *THRB* which are located in human chromosome 17 and 3, respectively (Sap et al., 1986, Weinberger et al., 1986). Studies to determine *Thr* gene expression and functions have been mostly conducted in mouse. *Thra1* mRNA is expressed in various tissues and organs. *Thra1* is important for brain development and cardiac activity (Gloss et al., 2001, Wallis et al., 2010). *Thrb1* mRNA is predominantly expressed in the liver and regulate T_3 effect on cholesterol metabolism (Gullberg et al., 2002). *Thrb2* mRNA is pituitary-specific and responsible for T_3-mediated negative feedback regulation by TRH neurons (Abel et al., 2001, Hodin et al., 1989).

Further studies on THR expression at the protein levels has been proven to be difficult due to the low expression of endogenous THR. In addition, there are no reliable antibodies against THR (Liu et al., 2013). The generation of new knock-in mice carrying hemagglutinin insertion endogenously tagging THR isoforms enables studies on THR expression at the protein levels (Minakhina et al., 2020, Pinto et al., 2017). A recent study on these mice revealed that THRA1 is the predominantly expressed THR protein isoform in all organs, except the liver. THRB1 is predominantly expressed in the liver, while THRB2 is only present in pituitary extracts. Furthermore, THRB2 expression is higher in female mice compared to male mice (Minakhina et al., 2020).

1.2.5. Krüppel-like factor 9 (KLF9)

Krüppel-like factor 9 (*Klf9*) gene, formerly known as basic transcription element binding (*Bteb*) protein 1, was initially isolated from rat liver (Imataka et al., 1992). *Klf9* activates genes that have repeated GC box in the promoter regions. This gene is highly expressed in brain, kidney, lung, and testis (Imataka et al., 1992). The direct regulation of T_3 on *Klf9* gene expression in mammals was first detected *in vitro* in the developing rat brain. This

action is mediated by *Thrb*. T_3 regulation on *Klf9* gene expression disappears in adult rat brain (Denver et al., 1999). The presence of TRE was later identified in the transcription start site of the mouse *Klf9* gene, explaining its regulation by T_3 (Denver and Williamson, 2009). *Klf9* has various roles in different cells. *Klf9* induction following T_3 exposure is important in oligodendrocyte regeneration (Dugas et al., 2012). Furthermore, *Klf9* gene expression can be induced following crosstalk between T_3/*Thr* and glucocorticoid signalling *in vitro* during mouse neural cell development (Gil-Ibanez et al., 2014). In addition, T_3-mediated *Klf9* gene induction stimulates *Dio1* activation in mouse liver cells (Ohguchi et al., 2008).

KLF9 is a pro-adipogenic transcription factor (Kimura and Fujimori, 2014, Pei et al., 2011). It is expressed in preadipocytes and adipocytes at mRNA and protein level. KLF9 involves in early and middle stage of adipogenesis by activating adipogenesis markers, such as CCAAT/enhancer-binding protein (C/EBP)*β* and PPARγ2, which are known to have *Klf9* gene binding sites on their promoter regions (Kimura and Fujimori, 2014, Pei et al., 2011).

Other than TRE, *KLF9*/*Klf9* gene contains several conserved antioxidant response elements, which are the binding sites for a major regulator of antioxidant defence, nuclear factor erythroid 2-related factor (Nrf2) (Zucker et al., 2014). A study in human lung fibroblasts and mouse embryonic fibroblasts showed that the presence of oxidative stressor such as H_2O_2 induces *KLF9*/*Klf9* gene expression via Nrf2 in the cells. This induction leads to ROS accumulation and can result in cell death (Zucker et al., 2014).

1.2.6. Hairless (Hr)

Mouse hairless (*hr*) gene located on chromosome 14 encodes an approximately 130 kDa nuclear transcription factor (Hr) (Cachon-Gonzalez et al., 1994). *hr* gene has an important role in the brain because TH directly mediates *hr* gene expression in developing rat brain (Thompson, 1996, Thompson and Bottcher, 1997). Hr protein interacts directly and specifically with THR (Thompson and Bottcher, 1997). However, the interaction between rodent Hr protein with unliganded THRs leads to the repression of their transactivation activity (Potter et al., 2001). This repressive role is established by interaction between Hr and histone deacetylase that modify chromatin structure to silence

gene transcription, which determine the role of Hr as a nuclear receptor corepressor (Potter et al., 2001).

Hr contains a zinc-finger-like domain that mediate selective direct DNA binding with p53 responsive element (Brook et al., 2017). This has implications for a crosstalk between Hr and p53 pathway that result in anti-apoptotic response of Hr by repressing p53 expression and pro-apoptosis events regulated by p53 (O'Driscoll and Bressler, 2010).

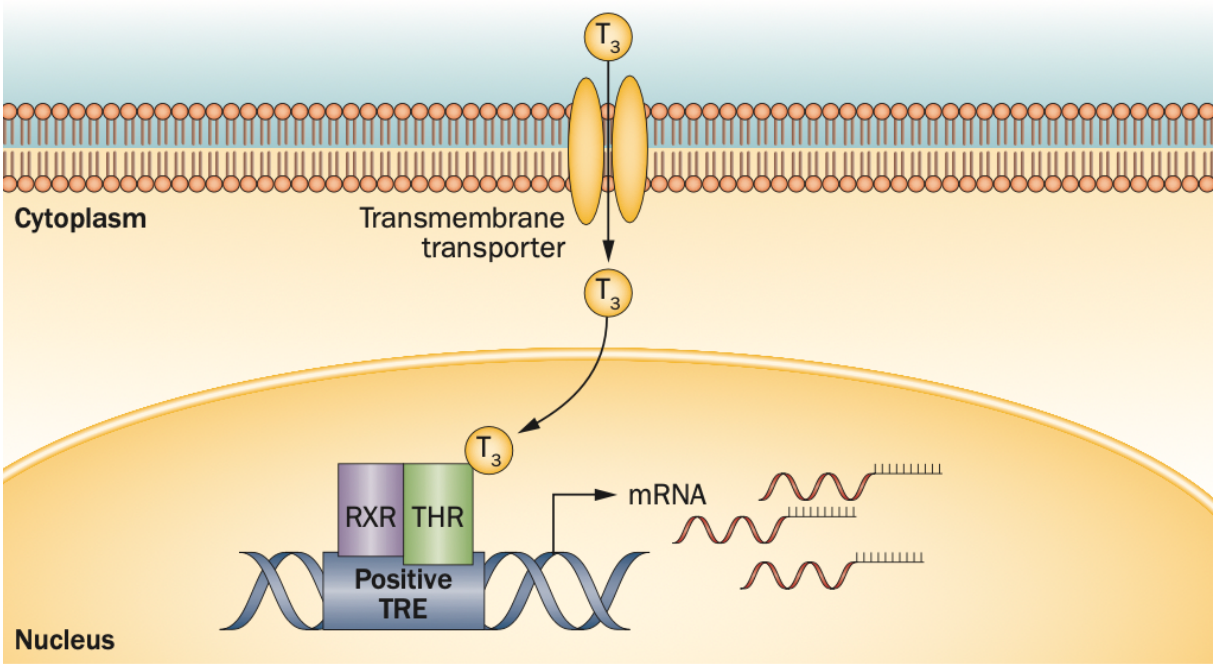

Figure 5. Overview of TH action. T_3 enters the cells via TH transporters, or is generated locally via cytoplasmic deiodinases. T_3 binds to THRs-containing dimers with RXR in the nucleus. This complex recognizes genomic TRE and regulates gene transcription (Ortiga-Carvalho et al., 2014).

1.2.7. TH receptor knockout (THR-KO) Mouse Models

The progress in understanding T_3 action *in vivo* mainly relies on observations performed on THR knockout (THR-KO) mice. Therefore, the design of THR-KO mice is crucial for determining the phenotype outcome (Flamant and Gauthier, 2013).

THRB-KO (*Thrb*$^{-/-}$) mice were generated by targeted disruption of *Thrb* gene (Forrest et al., 1996). These mice were viable and showed normal growth and weight gain. Moreover, *Thrb*$^{-/-}$ mice were fertile. However, the thyroid glands of *Thrb*$^{-/-}$ mice displayed a hyperactive state. Furthermore, the level of serum T_4 and free T_3 were elevated in *Thrb*$^{-}$

$^{/-}$ mice. The main feature of these mice was elevation of TSH despite high level of serum TH. This demonstrated a crucial function of *Thrb* in HPT axis regulation (Forrest et al., 1996).

The first attempt to generate THRA-KO (*Thra*$^{-/-}$) mice was performed by inactivating *Thra1* and *Thra2* transcripts expression, but *Thr*Δ*a* transcript was still being expressed (Fraichard et al., 1997). This experiment led to death in mice after weaning period. Furthermore, during the first two weeks of life, *Thra*$^{-/-}$ mice become increasingly hypothyroid and show growth arrest. In addition, small intestine and bones displayed a strongly delayed maturation (Fraichard et al., 1997). A comparison between *Thra*$^{-/-}$ (Fraichard et al., 1997) and *Thrb*$^{-/-}$ mice (Forrest et al., 1996) showed that the products of both *Thra* and *Thrb* genes are involved in the control of TSH production in different ways (Gauthier et al., 1999). Therefore, in the absence of *Thrb*, the products of *Thra* gene could also control TSH production (Gauthier et al., 1999).

The generation of an improved THR-KO mice (*Thra*$^{0/0}$) in 2001 resolved the problem of death after weaning period and severe impairment in intestinal maturation that occurred in the previous model (Gauthier et al., 2001). This mouse model was lacking of all known *Thra* isoforms. Furthermore, this mouse model revealed the role of *Thr*Δ*a* isoform in regulating intestinal development and mouse survival during weaning period (Gauthier et al., 2001).

1.2.8. Non-canonical TH signalling

Actions of TH that do not primarily involve the cell nucleus is called non-canonical TH signalling, or formerly known as non-genomic TH signalling (Davis et al., 2016). These TH actions were originally detected in mitochondria and cytoskeleton (Siegrist-Kaiser et al., 1990, Sterling et al., 1980). Non-canonical TH actions were described to be rapid in onset (within minutes or a few hours) and did not require gene transcription and protein synthesis (Siegrist-Kaiser et al., 1990, Sterling et al., 1980).

While T_4 has been viewed as a pro-hormone for T_3 and non-functional in canonical TH signalling, this hormone is required for some functions in non-canonical TH signalling (Siegrist-Kaiser et al., 1990). There have been various experimental studies that prove the existence of non-canonical TH signalling. A study in mature human erythrocytes showed that T_4 enhance Ca^{2+}-ATPase activity by 45% and T_3 by 33% in erythrocytes membrane.

This increased activity was reflected in ion transport function of the enzyme (Davis et al., 1983). Other experimental approaches were performed in cells lacking THR, such as HeLa cells (Lin et al., 1998, Lin et al., 1999a, Lin et al., 1999b, Shih et al., 2001). In HeLa cells, T_4 activates signal transduction of downstream proteins (Lin et al., 1998, Lin et al., 1999a, Lin et al., 1999b) and p53 phosphorylation (Shih et al., 2001).

The discovery of a plasma membrane TH receptor expressed by integrin $\alpha v\beta 3$ has helped further explain the non-canonical actions of TH (Bergh et al., 2005). In response to T_4, the αv monomer of integrin was internalized by cells and taken up by the nucleus where it functions as co-activator of hypoxia inducible factor-1a (*HIF-1A)* and *THRB1* genes (Lin et al., 2013). This indicated that non-canonical TH signalling also involves changes of gene expression (Lin et al., 2013). Therefore, there was a correction in the nomenclature suggesting a change of term from "non-genomic" to "non-canonical" TH actions (Flamant et al., 2017). In their review, Flamant and colleagues proposed four types of TH signalling based on THR requirement (Flamant et al., 2017). Type 1 is THR-dependent TH signalling with direct binding to DNA, which is the known canonical TH signalling; type 2 is THR-dependent TH signalling with indirect binding to DNA; type 3 is THR-dependent TH signalling without DNA binding; and type 4 is THR-independent TH signalling (Flamant et al., 2017).

The type 3 TH signalling has been thoroughly studied on knock-in mouse models ($Thra^{GS}$ and $Thrb^{GS}$) that lack of *Thra* and *Thrb* DNA binding ability (Geist et al., 2021, Hönes et al., 2017). Using the $Thra^{GS}$ and $Thrb^{GS}$ mouse models, Hönes and colleagues reported that several physiological effects of TH are preserved despite the absence of *Thra* and *Thrb* DNA binding (Hönes et al., 2017). They showed that heart rate, body temperature, blood glucose, and triglyceride concentration are regulated by non-canonical TH signalling (Hönes et al., 2017). Recently, a further study conducted by Geist and colleagues revealed that non-canonical TH-*Thra* actions on cardiovascular system induces vasodilation of mesenteric arteries via PI3K and endothelial NO-synthase (eNOS) activation (Geist et al., 2021).

1.3. TH and the immune system

It is known that there is a connection between endocrine and immune system (De Luca et al., 2020, Klein, 2021). Immune cells, such as monocytes, granulocytes, NK cells, mast

cells, and lymphocytes are considered as TH target cells (De Vito et al., 2011). Thus, changes of TH status in the circulation affect both innate and adaptive immune responses (Rubingh et al., 2020).

1.3.1. The Roles of TH in Innate Immune Responses

Neutrophils

Neutrophils are the most abundant circulating leukocytes and play an important role in pathogen killing (Kolaczkowska and Kubes, 2013). Furthermore, human neutrophils express additional elements required for TH metabolism, such as *MCT10*, *DIO1*, *DIO3*, and *THRA1* mRNA (van der Spek et al., 2016).

The exposure of neutrophils to T_3 for a short term has been associated with significant increase in ROS generation. Magsino and colleagues reported a significant upregulation in ROS generation accompanied by strong expression of markers for oxidative damage after treating healthy people with T_3 for 7 days (Magsino et al., 2000).

The effect of TH (T_4, T_3, and T_2) on neutrophil function was further confirmed by Mezosi and colleagues in an *in vitro* study (Mezosi et al., 2005). TH addition to the cell culture results in superoxide anion (O_2^-) production within a few minutes, suggesting a non-canonical action of TH in these cells. This action was mediated by G protein via PKC stimulation (Mezosi et al., 2005).

Furthermore, the presence and the protective role of DIO3 in neutrophils were reported for the first time by Boelen and colleagues (Boelen et al., 2009). The oxidative stress resulting from ROS generation increased DIO3 activity, which reduced intracellular T_3 content. This decrease in T_3 bioavailability gave protective roles to the cells, such as prolonging cell survival, extending antimicrobial abilities, and limiting extracellular tissue damage (van der Spek et al., 2016).

Macrophages

The presence of macrophages is crucial for tissue homeostasis and immunity (Wynn et al., 2013). In response to stimulations from their microenvironment, macrophages can perform polarization, which is a shifting between a pro-inflammatory (M1) and an anti-inflammatory (M2) phenotype (Sica and Mantovani, 2012). Macrophages lacking of Dio2 showed an impaired pro-inflammatory cytokine response to lipopolysaccharide (LPS)

stimulation (Kwakkel et al., 2014). Furthermore, macrophages derived from *Thra*$^{0/0}$ mice showed increased pro-inflammatory cytokines at baseline (Billon et al., 2014, Furuya et al., 2017), but reduced pro-inflammatory cytokine in response to LPS stimulation (Kwakkel et al., 2014). This indicated that locally produced T_3 acting via *Thra* is important in regulating inflammatory response of macrophages (Kwakkel et al., 2014). A recent study on macrophage cell line reported that *Thra* siRNA knockdown reduces M1 markers and increases M2 markers after polarization. This further indicated that intracellular T_3 signalling via *Thra* regulates inflammatory response of macrophages by inducing pro-inflammatory M1 polarization (van der Spek et al., 2018).

Dendritic Cells

Dendritic cells (DCs) are the most important APCs and main regulator of immune response (Banchereau and Steinman, 1998). The first study that examined the role of TH on DCs was conducted by Mooij and colleagues in 1994 (Mooij et al., 1994). In this study, they stimulated human peripheral blood monocytes with T_3 and T_4 and found that TH enhances monocytes maturation into DCs (Mooij et al., 1994). This mechanism is dependent on the production of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and TNFα (Mooij et al., 1994). The role of TH on DCs was further studied in patients, which shows the ability of TH in enhancing DCs proliferation and maturation (Dedecjus et al., 2011). L-T_4 administration to thyroidectomised patients increased the level of plasmacytoid and myeloid DCs in the peripheral blood (Dedecjus et al., 2011). Furthermore, CD86 expression, the marker for DC maturation, was enhanced following L-T_4 administration (Dedecjus et al., 2011). This result was subsequently confirmed in an *in vitro* study using DC cultures, which showed that T_3 stimulation increases CD86 expression (Dedecjus et al., 2011).

Mascanfroni and colleagues studied the signalling pathways involve in T_3-induced DC maturation (Mascanfroni et al., 2010). In bone marrow-derived DCs, treatment with T_3 led to Akt phosphorylation. Furthermore, T_3 treatment increased Thrb1 expression. Phosphorylated Akt interacted with Thrb1 in the cytoplasm and induced Thrb1 shuttling from cytoplasm to nucleus. These mechanisms showed the involvement of both canonical and non-canonical T_3 signalling pathway as well as the importance of Thrb1 in T_3-induced DC maturation (Mascanfroni et al., 2010). Moreover, T_3-Thrb1 activated NF-κB

pathway and stimulated DC maturation. Altogether, this study concluded that T_3 plays a role in DC maturation through Akt and NF-κb pathway under the control of Thrb1 (Mascanfroni et al., 2010).

Recently, Alamino and colleagues reported that *in vitro* T_3 treatment induces DCs to secrete pro-inflammatory cytokines (Alamino et al., 2019). Furthermore, they studied the effect of TH in transgenic mice expressing ovalbumin-specific T cell receptor. T_3 treatment to these transgenic mice induces DCs to express high level of IL-17 and IFNγ (Alamino et al., 2019). A further analysis on the isolated splenocytes showed that the cytokine production by DCs is mediated by increased production of $CD4^+$ and $CD8^+$ T cells (Alamino et al., 2019).

1.3.2. The Roles of TH in Adaptive Immune Reponses

The adaptive immune system mainly consists of T-cells and B-cells. TH is known to mediate the regulation of PKC content and cytokine production in lymphocytes (Klecha et al., 2006). Lymphocytes derived from hyperthyroid mice displayed higher T- and B-cell mitogen-induced proliferation compared to euthyroid mice (Klecha et al., 2006). Furthermore, total PKC content and mitogen-induced PKC translocation are higher in T and B cells from hyperthyroid mice (Klecha et al., 2006). In addition, LPS *in vivo* injection and *in vitro* re-stimulation increased IL-6 and IFNγ level in hyperthyroid cells (Klecha et al., 2006). These results were confirmed by Frick and colleagues who conducted a study on mice exposed to chronic stress. The circulating TH is reduced in these mice. In parallel to TH reduction, there is an impair in T cell activity (Frick et al., 2009). An *in vitro* study on T cells derived from mice exposed to chronic stress showed reduced expression of PKC isoforms (Frick et al., 2009). Recently it was reported that high levels of TH impairs the function of Tregs via programmed cell death 1 (PD-1) reduction (Zhong et al., 2021). T_3 treatment of human PBMCs reduces PD-1 expression in Tregs. Therefore, it is now known that hyperthyroidism plays an indirect role in Tregs dysfunction found in GD patients (Zhong et al., 2021).

The effect of TH in humoral immunity was indicated by alteration in B cells maturation and differentiation in response to high circulating level of T_3 (Bloise et al., 2014). T_3-treated mice showed increased number of splenic B lymphocytes. Furthermore, the percentage of pre-B cells and immature B cells were also enhanced in the bone marrow

of T_3-treated mice. This showed that excess T_3 induces B cells differentiation into plasma cells *in vivo* (Bloise et al., 2014).

1.4. Principal Issues Addressed in This Thesis

GO is a clinical manifestation of GD. Currently, TSHR is known as the shared autoantigen between GD and GO (Eckstein et al., 2006, Gerding et al., 2000). TSHR expressed by both thyroid cells (Smith & Hall, 1974) and OFs (Bell et al., 2000, Heufelder et al., 1993, Paschke et al., 1994, Stadlmayr et al., 1997, Wakelkamp et al., 2003) can be activated by TSAbs (Smith and Hall, 1974, Spitzweg et al., 1997) in GD and GO. Thus, it is currently known that TSAbs link the autoimmune reaction in GD and GO. A recent clinical study found that patients who show longer duration of hyperthyroid symptoms have higher possibility to develop GO (Wiersinga et al., 2018). In short, hyperthyroidism and GO are the main features of GD and occur together. However, the role of TH and hyperthyroidism in GO pathogenesis remains unknown, although a disease modifying role can be assumed given the role of TH in the immune system and in fibroblasts development and differentiation. This creates a missing link between these two important autoimmune hyperthyroid diseases. The aim of this thesis was to elucidate the role of TH in GO.

The experiments performed in the first part of this thesis would like to answer the questions whether TH is functional in OFs and whether TH plays an important role in GO. Furthermore, the experiments would like to determine the importance of THR in mediating TH actions in the OFs. The experiments performed in the second part of this thesis aimed to confirm if the *in vitro* effects of TH on OFs could be translated in WT mice.

CHAPTER 2: MATERIALS and METHODS

2.1. Orbital fibroblasts sources

2.1.1. Human orbital fibroblasts sources

Human orbital fibroblasts (OFs) were derived from GO patients and healthy control persons. GO orbital adipose tissues were obtained during orbital decompression procedures of severe GO patients. Orbital fat biopsies obtained during orbital surgery for other reasons were used as controls (Görtz et al., 2016a).

Patient ID	CAS	NOSPECS	GO Severity	Smoking Status
GO 112	1	4	Inactive	N.A
GO 118	5	12	Severe active	Smoker
GO 119	3	4	N.A	N.A
GO 128	2	9	N.A	Smoker

Table 1. Data on GO patient samples used in the cell culture experiment. CAS: Clinical Activity Score; NOSPECS: No physical signs or symptoms, only signs, soft tissue involvement, proptosis, extraocular muscle involvement, corneal involvement, sight loss; N.A: data not available

2.1.2. Mouse orbital fibroblasts sources

TSHR (TSHR mOFs) and β-Gal (β-Gal mOFs) mouse orbital fibroblasts were established from female BALB/c mice immunized with hTSHR A-subunit plasmid or β-Gal plasmid. All animals were sacrificed 9 weeks after the end of last immunization (Berchner-Pfannschmidt et al., 2016). All of the BALB/c mice used for cell culture experiments in this thesis were immunized and cultivated by Dr. med. Anke Daser (Department of Oto-Rhino-Laryngology, University Hospital Essen).

2.2. Mouse sources

2.2.1. Thyroid hormone receptor knocked-out (TR-KO) mice sources

Wild type (WT), *Thra*$^{0/0}$, and *Thrb*$^{-/-}$ mice in a C57BL/6 background were provided by Prof. Dr. med. Lars Möller and Dr. rer. nat. Sebastian Hönes (Department of Endocrinology, Diabetes, and Metabolism, University Hospital Essen). *Thra*$^{0/0}$ animals were generated on C57BL/6 background, by deleting all of the *Thra* isoforms (Gauthier et al., 2001). This mouse model is different from the previously known *Thra*$^{-/-}$, that still

retained *ThrΔa* isoforms (Fraichard et al., 1997). The latter model showed low survival after weaning period. *Thrb*$^{-/-}$ mice were lacking all known *Thrb* isoform. These animals were also generated in C57BL/6 background (Forrest et al., 1996).

2.3. Cell culture

2.3.1. Human orbital tissue isolation and OFs cell culture

GO orbital adipose tissues were isolated from patients with severe GO during orbital decompression procedures. Whereas, healthy orbital tissues were isolated from patients during orbital surgery for other reasons. All surgical procedures were performed by Prof. Dr. med. Anja Eckstein (Department of Ophthalmology, University Hospital Essen).
Tissues obtained from the orbital fat biopsies were washed with PBS and diced with a sterile scalpel blade. Next, they were placed in six-well culture plates containing 1 ml DMEM. Primary GO OFs (explant cultures) were grown in a humidified atmosphere of 10% CO_2 at 37^{o}C. Medium was replaced every 3-4 days. Once fibroblasts grew in adherent monolayers, they were transferred to 75-mL culture flasks by washing with phosphate buffer saline (PBS), followed by treatment with 0.25% trypsin/1.35 mM EDTA. Fibroblasts were serially passaged (Görtz et al., 2016a, Meyer zu Hörste et al., 2011). Fibroblasts between passage 2 and 6 were used in the experiment. Cell culture experiments, total RNA extraction, and cDNA synthesis of GO OFs were performed by Ms. Andrea Jaeger (Department of Endocrinology, Diabetes, and Metabolism, University Hospital Essen).

2.3.2. Mouse orbital tissue isolation and mOFs cell culture

All mice were sacrificed through carbon-dioxide euthanasia. After sacrifice, orbital tissue was dissected in a sterile biosafety cabinet under aseptic condition. All of the TR-KO mouse orbital tissue dissection for cell culture experiments were performed by Prof. Dr. med. Anja Eckstein. The oblique muscle from each orbit was directly transferred into a 35-mm culture dish, containing complete growth medium (Dulbecco's modified eagle medium, supplemented with 10% fetal calf serum (FCS); 10 mM HEPES (pH 7.4); 2 mM L-glutamine; 1 mM sodium pyruvate; 100 U/ml penicillin, and 100 μg/ml streptomycin – referred as "DMEM" for the remaining of this chapter). Primary mOFs were grown in a humidified atmosphere of 10% CO_2 at 37^{o}C. After one week, medium was replaced

with fresh DMEM. Cell growth was monitored and medium was replaced three times in a week.

Orbital tissues were grown in the tissue culture dish for approximately 4 weeks to allow mOFs growing out of the tissues. After mOFs were reaching 90% confluency, they were transferred to 25-mL cell culture flasks following the steps described below. All DMEM were discarded from the dishes. 1 mL PBS was added to the culture dishes twice to completely wash non-adherent mOFs. 1 mL of 0.05% trypsin solution was applied to detach mOFs from culture dishes. All mOFs were shortly incubated at 37^{o}C not exceeding 5 minutes. mOFs were quickly observed under the microscope to make sure that approximately 95% of mOFs were detached from the flask. 1 ml DMEM was added to stop the trypsinization reaction and mixed with the mOFs solution by pipetting. 4 mL DMEM were further added to mOFs cultures. Samples were incubated at 37^{o}C and mOFs growth was monitored 3 times in a week. When mOFs cultures formed approximately 80% confluent adherent monolayers, they were further transferred to 75-mL flasks and serially passaged. mOFs between passage 5 and 7 were used for the experiment. The remaining mOFs were kept frozen in DMEM with 10% DMSO solution. They were incubated on ice for 20 minutes, and stored in -80^{o}C for one week before being transferred to the liquid nitrogen for long-term storage.

2.3.3. Thyroid hormone (T_3) stimulation to OFs cell cultures

5 x 10^5 cells/mL OFs were seeded to each well in 6-well plates. After OFs reached approximately 95% confluence, medium was replaced by DMEM with 10% thyroid hormone (TH)-deficient FCS to render OFs hypothyroid. TH-deficient FCS was obtained following a method described by Samuels and colleagues (Samuels et al., 1979). In summary, 500 g resin was washed 6 times with 2 L H_2O and 25 mL of the washed resin was autoclaved after drying. 500 mL FCS was added to the resin (Amberlite® IRA-400 chloride form, Sigma-Aldrich) and stirred for 24 hours at 37^{o}C. 50 mL aliquots were stored at -20^{o}C after sterile filtration of TH-depleted FCS. The TH-depleted FCS aliquots were obtained from Ms. Andrea Jaeger, Department of Endocrinology. After being rendered hypothyroid for 48 hours, OFs were stimulated with 100 nM T_3 or NaOH solvent for 24 hours. Total RNAs were extracted from OFs and the concentrations were

determined. T_3 stock was prepared as 1 mM solution diluted in 40 mM NaOH containing 0.02% BSA. T_3 and NaOH aliquots were prepared by Dr. rer. nat Sebastian Hönes.

2.4. Quantitative reverse transcription PCR (RT-qPCR) preparation

2.4.1. Total RNA extraction from OFs cell cultures

Total RNAs were isolated using QIAGEN RNeasy™ mini kit, following the manufacturer's instruction. Cells in each well were detached using cell-scrapers and lysed by adding 350 µL *RLT-buffer* (containing 1% β-mercaptoethanol). One volume of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. 700 µL of the sample, including any precipitate that might have formed, were transferred to an RNeasy™ spin column placed in a 2-mL collection tube. Cells were centrifuged for 15s at 10,000 rpm. Flow-through was discarded. 350 µL *RW1 -buffer* was added to the spin column and centrifuged for 15s at 10,000 rpm to wash the spin column membrane. The flow-through was discarded. To eliminate genomic DNA contamination, on-column DNase digestion was performed. A mixture of 10 µL DNase I stock solution and 70 µL *RDD- buffer* were prepared for each sample and mixed gently by inverting the tube before use. 80 µL of DNase I incubation mix was added directly to the RNeasy spin column membrane, followed by a 15-minutes incubation at room temperature. 350 µL *RW1-buffer* was added further to RNeasy™ spin column and centrifuged for 15s at 10,000 rpm. Next, 500 µL *RPE- buffer* was added to the spin column and centrifuged for 15s at 10,000 rpm to wash the spin column membrane. The flow-through was discarded. For the final washing of the spin column membrane, 500 µL *RPE- buffer* were added one more time and centrifuged for 2 minutes at 10,000 rpm. The long centrifugation was performed to dry the spin column membrane and ensured that no ethanol was carried over during RNA elution. Subsequently, the spin column was placed in a new 2-mL collection tube and centrifuged for 1 minute at full speed. Finally, the spin column was placed in a new 1.5-mL collection tube. 40 µL RNase-free water was directly added to the spin column membrane and centrifuged for 1 minute at 10,000 rpm to collect the eluate. RNA concentration and purity were determined using EPOCH® spectrophotometer (BioTek).

2.4.2. Total RNA extraction from mOFs cell cultures

Total RNAs were isolated using QIAGEN RNeasy™ mini kit, following the procedures mentioned in section 2.4.1.

2.4.3. Total RNA extraction from mouse orbital tissue

Each orbital tissue sample was transferred to a sterile 14-mL tube that had been added with 1 mL Trizol reagent. The samples were then homogenized with shredder and incubated for 5 minutes at room temperature. The samples were centrifuged for 10 minutes at 11,000 rpm and 4^{o}C. The lysates were transferred to new 2-ml tubes. 400 µL chloroform was added and vortexed. Next, the samples were incubated for 3 minutes at room temperature. Samples were further centrifuged for 30 minutes at 4,500 rpm and 4^{o}C. After the centrifugation there were three phases observed in the samples. The RNA, the uppermost phase, was transferred to a new tube. 1.5 volume of 100% EtOH was added and mixed by inverting the tube. The samples were then transferred to the RNeasy spin column. The final steps were performed following QIAGEN RNeasy™ mini kit protocol as mentioned in section 2.4.1. RNA concentration and purity of mouse orbital tissue mRNA was measured in EPOCH® microplate spectrophotometer (BioTek).

2.4.4. Total RNA extraction from mouse liver tissue

30 mg of frozen liver tissue was excised using forceps on dry ice to prevent sample degradation. The sample was transferred to a sterile 14-mL tube that had been added with 600 µL *RLT- buffer*. This procedure was performed on ice. Next, the sample was homogenized using the shredder. The lysate was then centrifuged for 1 minute at 3000 rpm and transferred to a QiaShredder column. Subsequently, the lysate was centrifuged for 2 minutes at the maximum speed (13,300 rpm). 0.5 volume of 100% EtOH was added to the flow through and inverted for 10 times with the cap closed. 650 µL of the sample was transferred to RNeasy column and centrifuged for 15s at 13,300 rpm. This step was repeated until there were no lysates stick to the column anymore. The column was placed in a new 2-mL tube. The next steps were performed according to QIAGEN RNeasy™ mini kit protocol mentioned in section 2.4.1. Finally, RNA concentration and purity were measured using NanoDrop™ 2000 / 2000c spectrophotometer (ThermoFisher Scientific).

2.4.5. cDNA Synthesis

0.5 µg of total RNA were reverse transcribed into cDNA using Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase (Promega) and Oligo-dT primer. Briefly, 2.5 µL Oligo-dT was added to 0.5 µg RNA and the volume was brought up to 12 µL by adding diethyl pyrocarbonate (DEPC) water. The mixture was incubated at 68^{o}C for 10 minutes, then immediately placed on ice. 13 µL of cDNA-synthesis Master Mix consists of 5 µL 5x M-MLV reverse transcriptase (RT) Buffer, 5 µL NTPs, 2.5 µL DEPC water, and 0.5 µL M-MLV RT enzyme were added. The mixture was incubated at 45^{o}C for 90 minutes, followed by 30 minutes at 52°C. The reaction was terminated by incubation at 56^{o}C for 15 minutes. 1 µg of total RNA from pooled RNA samples was used for the efficiency control.

Following the incubation, each cDNA sample was diluted with DEPC water to reach the concentration of 1 ng/µL and further used for qPCR assay. The efficiency control was first diluted with DEPC water to 10 ng/µL, then serially diluted 1:2 until reaching the concentration of 1.25 ng/µL.

2.4.6. RT-qPCR assay

2.5 µL cDNA was mixed into 7.5 µL Master Mix (consist of 5 µL SYBR Green I Master Mix, 0.05 µL forward and reverse primers (10pmol/µL), and 2.4µL nuclease free water) in a white bottom 96-well plate. The RT-qPCR assay was performed in a light cycler LC480® (Roche) machine with the setting as follows: initial melting at 95^{o}C for 5 minutes, 40 cycles of amplification at 95^{o}C for 15 seconds, annealing at 60^{o}C for 10 seconds, and final denaturation at 72^{o}C for 20 seconds. A melting curve analysis was performed to detect unspecific RT-qPCR products and the formation of primer dimers. Peptidylprolyl isomerase A (*PPIA*/*Ppia)* was used as the reference gene for all experiments. All primers included in Table 1 and Table 2 were designed and optimized by Dr. rer. nat Sebastian Hönes.

Gene	Forward	Reverse	Accession No.
Ppia	CTT GGG CCG CGT CTC CTT CG	GCG TGT AAA GTC ACC ACC CTG GC	NM_008907.7
Thra	GAA AAG CAG CAT GTC AGG GTA	GGA TTG TGC GGC GAA AGA AG	NM_001313983.1
Thrb	GGA CAA GCA CCC ATC GTG AA	ACA TGG CAG CTC ACA AAA CAT	NM_001113417.1
Klf9	GAA ACA CGC CTC CGA AAA GAG	AGC GCG AGA ACT TTT TAA GGC	NM_010638.5
Mct8	CTC CTT CAC CAG CTC CCT AAG	ACT TCC AGC AGA TAC CAC ACC	NM_009197.2
hr	CCC TGA CCA CCG TAT GCT TT	CCA CAC CGT CTA AGT TCC CC	NM_001379479.1

Table 2. List of qPCR primers to detect mouse gene expression

Gene	Forward	Reverse	Accession No.
PPIA	AAC GTG GTA TAA AAG GGG CGG	CTG CAA ACA GCT CAA AGG AGA C	NM_021130.5
THRA	CCA CGC CAT CTT TGA ACT GG	GAG CGG TCT GTT GAC ATT AGC	NM_199334.5
THRB	GCG ATT TCC TTC TGG TTG GC	ACT GTT GGG AGT CAT AGG TTA GT	NM_000461.5
MCT8	AGC TCC TTC ACC AGC TCC CTA AGC	TCC TCC ACA TAC TTC ATC AGG TG	NM_006517.5
KLF9	CTC CCA TCT CAA AGC CCA TTA C	TGA GCG GGA GAA CTT TTT AAG G	NM_001206.4

Table 3. List of qPCR primers to detect human gene expression

2.5. *In vivo* experiments

Animal study approval

All animal experiments performed in this study were approved by Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen (LANUV).

2.5.1. Induction of hypothyroidism

In vivo studies of T_3 effect in mice required the animals to be rendered hypothyroid to ensure low and comparable TH concentration within different genotypes (Bianco et al., 2014). In this study, hypothyroidism was achieved in mice by administering MMI water (consist of 0.5% (5 g/L) sodium perchlorate; 0.05% (400 mg/L) 2-mercapto-1-methylimidazole (MMI); and 0,3% (3 g/L) saccharine diluted in warm tap water) and iodine-deficient diet (TD.95007, Harlan-Teklad, USA) for three weeks (Kerp et al., 2019).

2.5.2. Long term and short term T_3 treatment to WT C57BL/6 mice

Male C57BL/6 mice were used in this experiment. All mice were rendered hypothyroid for 3 weeks by modifying the content of their drinking water and diets following the protocol mentioned in section 2.5.1. Fresh batches of drinking water were given to all mice 2 times in a week throughout the experiment period. After 3 weeks, the mice were divided into 3 groups: MMI; long term T_3; and short term T_3 (n = 4 per group). Body weights were recorded 3 times -- at the beginning of the experiment, on the third week of

the experiment, and at the end of the experiment. Long term T_3 group received 0.5 µg/mL T_3 in the drinking water for 7 days. Animals in the MMI group were used as controls. Short term T_3 group received a single 20 µg/100 g body weight T_3 i.p injection. 1 mM T_3 stock solution was prepared in 40 mM NaOH containing 0.02% BSA. T_3 stock solution was diluted in sterile PBS for injection. T_3 i.p injection was performed by Dr.rer.nat Sebastian Hönes. Mice in the short term T_3 group were sacrificed 6 hours after injection. Whereas, mice in the other groups were sacrificed 7 days after start of T_3 treatment. All mice were sacrificed using isoflurane. Blood was withdrawn through heart puncture from the right ventricle. Then, mice were perfused via the left ventricle with 10 mL PBS/heparin (5 U/ml heparin) followed by a second perfusion with 10 mL PBS. The blood withdrawal was performed by Ms. Mareike Horstmann (Molecular Ophthalmology Laboratory, University Hospital Essen).

2.5.3. Tissue sampling for histological and biochemical analysis

After sacrifice, liver tissue as well as orbital tissue were isolated and shock-frozen in liquid nitrogen. Samples were stored in -80^{o}C for further histological and biochemical analysis. Orbital and liver tissue isolation was performed by Dr.rer.nat Svenja Philipp and Ms. Mareike Horstmann (Molecular Ophthalmology Laboratory, University Hospital Essen).

2.5.4. Serum preparation

Blood was collected in Microvette® tubes through heart puncture and incubated on ice for 30 minutes. Subsequently, the blood was centrifuged at 4^{o}C 13,400 rpm for 20 minutes. Next, serum was aliquoted and stored at -80^{o}C.

2.6. Analysis of serum parameters

2.6.1. Free T_3 (FT_3) protein concentration determination with ELISA assay

Before starting the assay, H_2O_2/TMB solution was prepared by mixing Color Reagent A with Color Reagent B (1:1 dilution). Both reagents were provided with the DRG FT_3 ELISA kit. 50 µL standard and 50 µL control were pipetted into the microplate wells. Next, 25 µL of each serum sample were pipetted and then topped up with 25 µL of "0" standard. 100 µL of FT_3-enzyme conjugate reagent was added to each microplate well

and mixed for 20 to 30s. The microplate was incubated for 60 minutes at room temperature in the dark. Subsequently, the microplate content was emptied and rinsed five times with deionized water. 200 µL H_2O_2/TMB solution was added to each microplate well and mixed for 10s. The microplate was left for another 20-minutes incubation at room temperature in the dark. Lastly, 50 µL stop solution was added to each microplate well and mixed for 30s. The absorbance of each sample was read at 450 nm with a microtiter plate reader.

2.6.2. Total T_4 (TT_4) protein concentration determination with ELISA assay

Before starting the assay, the T_4-HRPO conjugate reagent was prepared by adding 100 µL T_4-HRPO conjugate concentrate to 1 mL T_4 conjugate diluent. 25 µL standards and controls were pipetted into the microplate wells. Next, 10 µL of each serum sample were pipetted and then topped up with 15 µL of "0" standard. 100 µL T_4-HRPO conjugate reagent was added into each well and mixed for 30s. The microplate was incubated at room temperature for 60 minutes in the dark, then all of the content was emptied and rinsed for 5 times with deionized water. 100 µL TMB reagent was dispensed to each microplate well and mixed gently for 10s. The microplate was once more incubated for 20 minutes at room temperature, in the dark. Finally, 100 µL stop solution was added to each microplate well and mixed for 30s. The absorbance of each sample was read at 450 nm with a microtiter plate reader.

2.7. Animal Backcrossing and Genotyping

2.7.1. Animal Backcrossing

Male C57BL/6 *Thra*$^{+/0}$ or *Thrb*$^{+/-}$ mice were backcrossed to female WT BALB/c mice for 8 generations. Following the last backcrossing, *Thra*$^{+/0}$ or *Thrb*$^{+/-}$ mice were mated with their heterozygous littermates to obtain homozygous (*Thra*$^{0/0}$ or *Thrb*$^{-/-}$) BALB/c. All backcrossing procedures were performed by Central Animal Laboratory (ZTL) personnel at the University Hospital Essen.

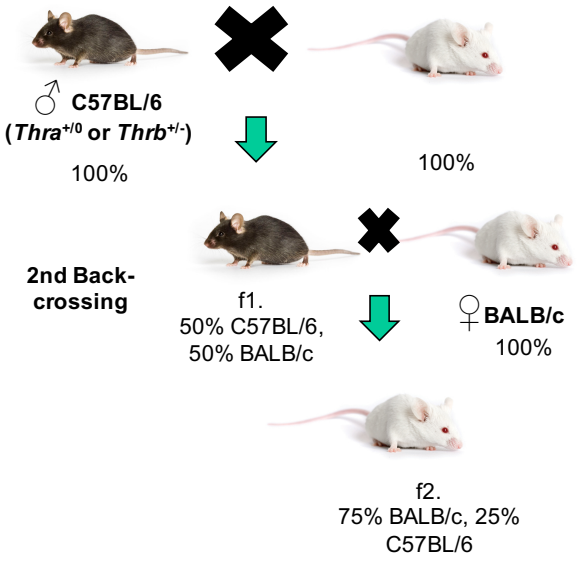

Figure 6. Mouse backcrossing scheme

A mouse model with BALB/c background was generated in this thesis to study TH effect in GO. *Thra*$^{+/0}$ or *Thrb*$^{+/-}$ was backcrossed to female WT BALB/c mice to transfer BALB/c genotype to the *Thra*$^{+/0}$ or *Thrb*$^{+/-}$ mice. The purpose of using female mice was to accelerate genotype transfer (Grove et al., 2016). By the end of the eighth backcrossing, *Thra*$^{+/0}$ or *Thrb*$^{+/-}$ mice carrying nearly 100% BALB/c characteristics were obtained. The total animals generated throughout the eight generation of backcrossing were as follows: 23 male *Thra*$^{+/0}$;19 female *Thra*$^{+/0}$; 21 male *Thra*$^{+/+}$; 23 female *Thra*$^{+/+}$; 14 male *Thrb*$^{+/-}$; 22 female *Thrb*$^{+/-}$; 17 male *Thrb*$^{+/+}$; and 19 female *Thrb*$^{+/+}$ (Table 4 and Table 5). Subsequently, female heterozygous littermates were mated with male heterozygous littermates to generate *Thra*$^{0/0}$ or *Thrb*$^{-/-}$ BALB/c for further *in vivo* study. However, due to the COVID-19 pandemic, the *in vivo* study using this animal model was postponed and therefore the results could not be included in this thesis.

2.7.2. Genotyping of mouse strains

DNA was extracted from ear biopsy samples obtained from littermates resulting from every backcrossing. 75 µL of lysis buffer was added to the samples prior to boiling for 45 minutes at 95^{o}C. An equal volume of neutralization buffer was then added to the samples. The lysis buffer stock was made of 125 µL NaOH, 100 µL EDTA-disodium, and 4.8 mL distilled water. The neutralization buffer stock was made of 200 µL Tris-HCl and 4.8 mL distilled water. A thorough vortexing was performed to homogenize the

extracted DNA. 2 µL of the extracted DNA was used for genotyping PCR. For *Thra*$^{+/0}$ and *Thra*$^{0/0}$ genotyping, the PCR Master Mix consists of 2 µL 10x reaction buffer; 1.5 µL 50 mM $MgCl_2$; 0.4 µL 100 nM dNTPs; 0.5 µL forward and reverse primers (5 pmol/µL each); 0.4 µL Taq polymerase; and 11.7 µL DEPC water. For *Thrb*$^{+/-}$ and *Thrb*$^{-/-}$ genotyping, the PCR Master Mix consists of 4 µL 5x reaction buffer; 3 µL 25mM $MgCl_2$; 0.8 µL 50 mM dNTPs; 0.5 µL forward and reverse primers (5 pmol/µL each); 0.05 µL Taq polymerase; and 8.2 µL DEPC water. 18 µL of the Master Mix was distributed to each PCR tube. 2 µL of extracted DNA from each sample was then added to the respective tube. The PCR was performed with the setting as follows: initial denaturation at 95^{o}C for 3 minutes, 40 cycles consisting of denaturation at 95^{o}C for 30s, annealing at 60^{o}C for 20s, and elongation at 72^{o}C for 30s. A final extension step was done at 72^{o}C for 3 minutes. All of the primers used for genotyping included in Table 6 were designed and optimised by Dr.rer.nat Sebastian Hönes.

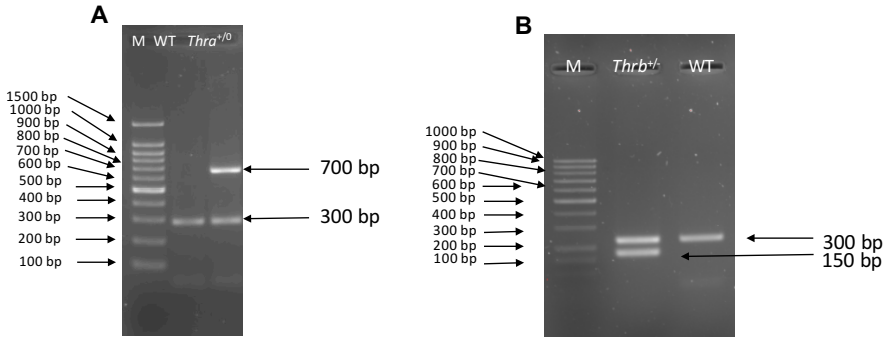

Figure 7. Representative gel electrophoresis visualization images of genotyping from (A) DNA derived from TRα-KO (*Thra*$^{+/0}$) and WT mice and (B) DNA derived from TRβ-KO (*Thrb*$^{+/-}$) and WT mice. Arrows indicate DNA size of each samples. Electrophoresis was performed using 2% gel agarose. M = 100 bp DNA marker (Qiagen GelPilot 100 bp Plus Ladder, Cat No. 239045).

Backcrossing	Breeding pair #	*Thra* +/0		WT	
		Male	Female	Male	Female
f1	1	1	2	0	0
	2	0	3	2	5
f2	1	2	3	1	2
f3	1	0	1	2	0
	2	2	2	1	1
f4	1	1	2	0	0
	2	2	4	1	2
f5	1	2	0	2	3
	2	2	1	0	0
f6	1	1	0	1	4
f7	1	3	1	0	3
f8	1	2	0	6	1
	2	2	0	4	2
	3	3	0	1	0

Table 4. Overview of littermates from *Thra*$^{+/0}$ to BALB/c backcrossing for eight generation (f1-f8 = littermates for each generation).

Backcrossing	Breeding pair #	*Thrb* +/-		WT	
		Male	Female	Male	Female
f1	1	4	1	1	1
f2	1	2	3	1	4
	2	1	1	0	0
f3	1	1	0	1	0
f4	1	1	3	5	0
	2	0	1	2	2
f5	1	0	1	0	5
	2	0	1	0	0
f6	1	1	0	2	1
	2	0	0	2	2
f7	1	2	3	1	0
f8	1	1	4	0	2
	2	1	4	2	2

Table 5. Overview of littermates from *Thrb*$^{+/-}$ to BALB/c backcrossing for eight generation (f1-f8 = littermates for each generation).

Genotype	Gene	Sequence	Product Size (bp)
TR$\alpha^{+/+}$	*Thra* WT-F	TCC TGA AGA GTG GGA CCT GAT	300
	Thra WT-R	GCC TTC TTA CCA GGA ATT TTC GC	
TRα^{00}	*Thra* KO-F	GCA TCG CCT TCT ACT GCC TT	660
	Thra KO-R	GAG GAT GAT CTG GTC TTC GCA A	
TR$\beta^{+/+}$	*Thrb* WT-F	CCT CTC ACC TTT CTA CTT TGC	250
	Thrb WT-R	CAG GAA TTT CCG CTT CTG CTT	
TR$\beta^{-/-}$	*Thrb* KO-F	TGA ACC TAT TAT CTC GGG TCT TTC TC	150
	Thrb KO-R	GCC TCT TCG CTA TTA CGC CA	

Table 6. List of primers for mouse genotyping

20 µL of *Thra*$^{+/0}$ PCR products were mixed with 2 µL of 2x Blue Juice™ loading buffer before proceeding to gel electrophoresis which was performed to separate the DNA of each sample based on the size. *Thrb*$^{+/-}$ PCR products were loaded directly to the wells on agarose gel. For gel electrophoresis, 2% agarose gel (w/v in 1x TAE buffer) was used. Gel electrophoresis was performed for 40 minutes at 100V. 4.5 µL 100 bp GelPilot DNA ladder was added to determine the DNA band size. Gel visualization was performed in VersaDoc 4000 MP machine (Bio-Rad Laboratories Inc, USA).

2.8. Statistics and Software

One-way ANOVA with Tukey's post-hoc test was used for quantitative reverse transcription PCR (RT-qPCR) analysis of TH-responsive genes expression in OFs, RT-qPCR analysis of TH-responsive genes in mouse liver mRNA, and serum analysis. Unpaired t-test with Welch's correction was used for RT-qPCR analysis of TH-responsive genes in mouse orbital mRNA. Two-way ANOVA with Sidak's multiple comparisons test was used for analysis of percent mouse body weight changes. Statistically significant differences between groups were marked with $*P < 0.05$; $**P < 0.005$; $***P < 0.001$; and $****P < 0.0001$. Statistical analysis and graph plotting were performed using GraphPad Prism version 8 (GraphPad Software, USA)

RT-qPCR statistical analysis

Relative gene expression calculation was performed following the efficiency ($E^{-\Delta\Delta Cq}$) corrected method according to the quantification cycle (C*q*) values, which is the fractional PCR cycle at which the target is quantified in a given sample, obtained from RT-qPCR assay (Lefever et al., 2009, Pfaffl, 2001). The results were then normalized to C*q* values of the reference gene, *PPIA*/*Ppia*. All gene expression data were presented as mean of the gene expression ± SEM, relative to the untreated control unless otherwise stated. Only C*q* values under 35 were used for analysis and relative gene expression calculation.

Primer design software and conditions

Primer BLAST (NCBI, Bethesda, USA) was used to design primers for genotyping and qRT-PCR. Primers should span exon-exon junction, if possible. The amplicon size was

adjusted to 70-200 bp. The melting temperature of 60 ± 3^{o}C was preferred. All of the primers used in this thesis were designed and optimized by Dr.rer.nat Sebastian Hönes.

CHAPTER 3: RESULTS

3.1. Evaluation of TH responsive genes in human OFs

THRA and *THRB* are the genes that encode thyroid hormone receptor isoforms (THRA and THRB), ligand-dependent transcription factors. *THRA* and *THRB* bind thyroid hormone (TH) responsive elements (TRE) in the presence and absence of TH to mediate TH-target genes expression (Anyetei-Anum et al., 2018). Despite being present in various tissues, *THR* genes expression has not been studied in human OFs. To evaluate *THR* genes in human OFs, their expression was determined in OFs derived from GO patients (GO OFs) and healthy control by RT-qPCR analysis and functional responsiveness assessed to T_3. GO OFs and control OFs were used at low passage number, not exceeding passage number 6.

The initiation of biological processes in human body system requires interaction of multiple transcription factors that regulate different signals. T_3 has been reported to work through *THRA* and *THRB* to induce *KLF9* gene expression (Cvoro et al., 2015). *KLF9* gene is well known to respond strongly to T_3 and hence was used as a control gene for evaluating responses to T_3 in GO OFs.

TH plays a role in metabolism by acting locally at the cellular level through a canonical signalling pathway. For that purpose, TH needs to be transported across the cell membrane. There are at least five known transmembrane transporter families, which include the organic anion transporters (OATPs, SLC10, and SLC17), LATs, and MCTs (Groeneweg et al., 2020). *MCT8* gene expression was selected to be observed in this thesis because it encodes a highly specific and effective TH transporter (Friesema et al., 2003).

3.1.1. *THRA* was the predominantly expressed *THR* in GO OFs

According to the RT-qPCR analysis, *THRA* mRNA was detected in GO OFs with the average *Cq* value of 26.4. Whereas, *THRB* mRNA was detected in GO OFs with the average *Cq* value of 29.4. *THRA* as well as *THRB* gene in GO OFs were not responsive to T_3 treatment (Figure 8, Panel A and B). Interestingly, T_3 upregulated *THRB* gene expression in control OFs (Figure 8, Panel B).

To determine the predominantly expressed *THR* isoform gene in GO OFs, relative expression of *THRB* gene was compared to that of *THRA* gene according to their C*q* values. Based on the comparison, *THRA* was the predominantly expressed *THR* isoform in GO OFs and control OFs (Figure 9, Panel A and B).

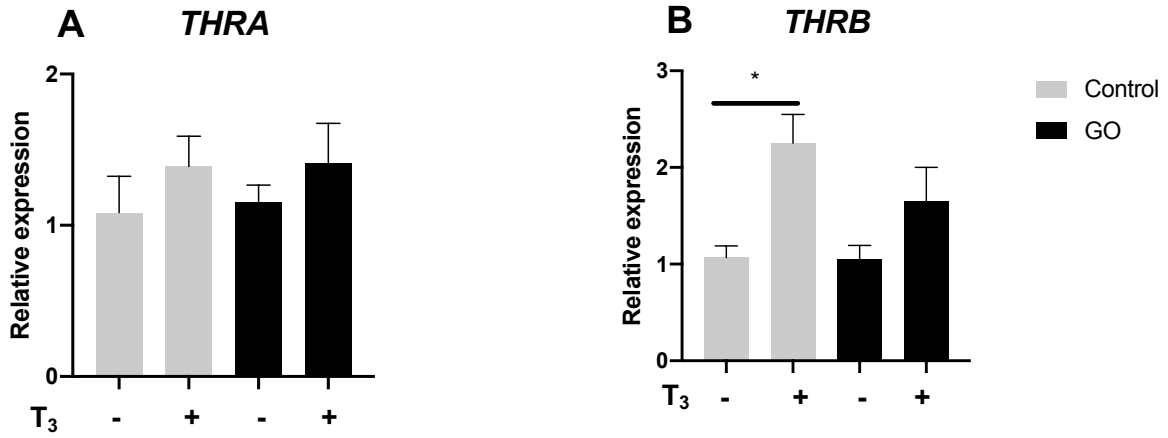

Figure 8. (A) *THRA* and (B) *THRB* gene expression in GO or control OFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated control. All data were normalized to *PPIA* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test; *$P < 0.05$).

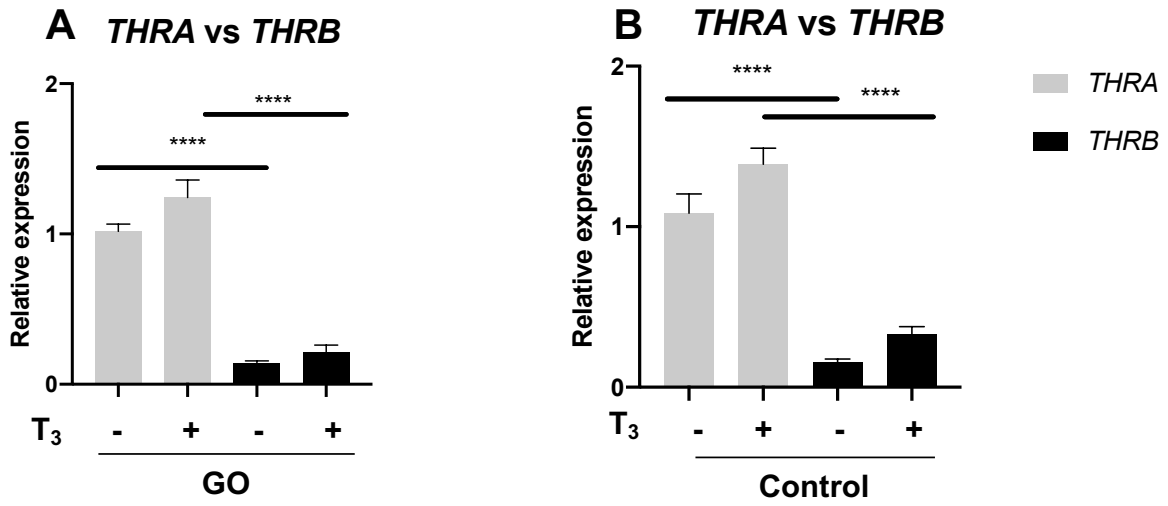

Figure 9. Comparison between *THRA* and *THRB* gene expression in (A) GO OFs and (B) control OFs. Data are presented as mean of *THRA* or *THRB* gene expression ± SEM, relative to *THRA* gene expression in untreated GO OFs or *THRA* gene expression in untreated control. All data were normalized to *PPIA* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test. ****$P < 0.0001$).

3.1.2. T_3 treatment upregulated *KLF9* expression in GO OFs

As shown in Figure 10, *KLF9* gene expression was significantly upregulated following T_3 treatment in GO OFs. The effect of T_3 treatment on *KLF9* expression in GO OFs was comparable to that in control.

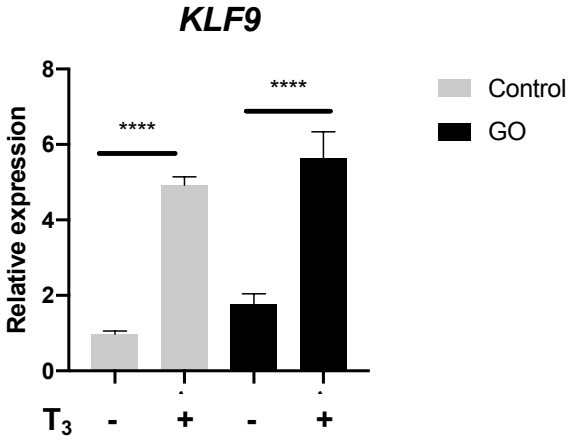

Figure 10. *KLF9* gene expression in GO or control OFs with or without 100 nM T_3. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated control. All data were normalized to *PPIA* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test; **** $P < 0.0001$).

3.1.3. T_3 treatment did not alter *MCT8* gene expression in GO OFs

According to the RT-qPCR analysis, *MCT8* mRNA was detected in GO OFs with the average *Cq* value of 28.8. The level of *MCT8* gene expression in GO OFs was comparable to those of control OFs. Furthermore, T_3 treatment did not alter the level of *MCT8* gene in both OFs (Figure 11).

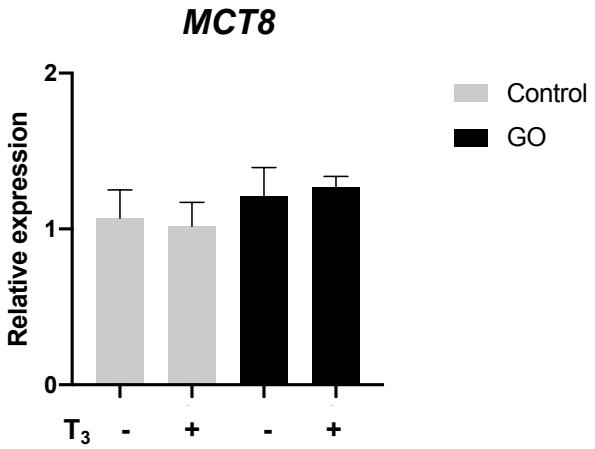

Figure 11. *MCT8* gene expression in GO or control OFs with or without 100 nM T_3. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated control. All data were normalized to *PPIA* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test).

3.2. Evaluation of TH responsive genes in experimental GD/GO mouse OFs (mOFs)

In the previous section, it was shown that GO OFs expressed TH-responsive genes. In these set of experiments, mOFs derived from the established experimental female GD/GO mouse model generated by immunization with human TSHR A-subunit plasmid through electroporation (TSHR mOFs) were used. As controls, mOFs derived from female BALB/c mice immunized with β-Gal plasmid (β-Gal mOFs) were used in these experiments.

Firstly, *Thra* and *Thrb* gene expression was observed in TSHR mOFs. Subsequently, the presence of two known TH-responsive genes, *Klf9* and *hairless* (*hr*) (Thompson, 1996, Thompson and Bottcher, 1997), and their response towards T_3 treatment were tested to determine the functionality of *Thra* and *Thrb* in TSHR mOFs. Finally, *Mct8* gene expression were examined to decide whether T_3 treatment to TSHR mOFs and/or hTSHR immunization play a role in the gene expression.

3.2.1. *Thra* was the predominantly expressed *Thr* in TSHR mOFs

According to the RT-qPCR analysis, *Thra* and *Thrb* mRNA were also detected in experimental TSHR mOFs and β-Gal mOFs. The addition of T_3 to the cultures did not alter the expression of both *Thra* and *Thrb* (Figure 12, Panel A and B).

To determine which of the *Thr* gene was predominantly expressed in mOFs, *Thra* and *Thrb* relative gene expression were compared according to their C*q* values. *Thra* gene was the predominantly expressed *Thr* in TSHR mOFs as well as in β-Gal-mOFs (Figure 13, Panel A and B).

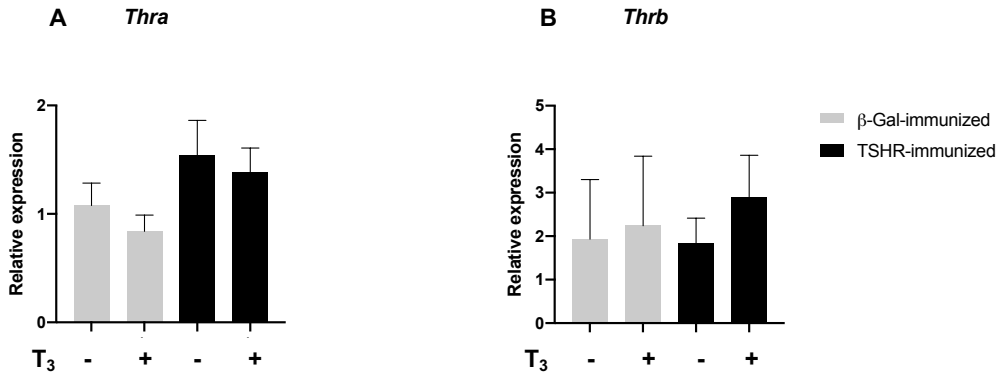

Figure 12. (A) *Thra* and (B) *Thrb* gene expression in TSHR-immunized or *β*-Gal mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated *β*-Gal mOFs. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test).

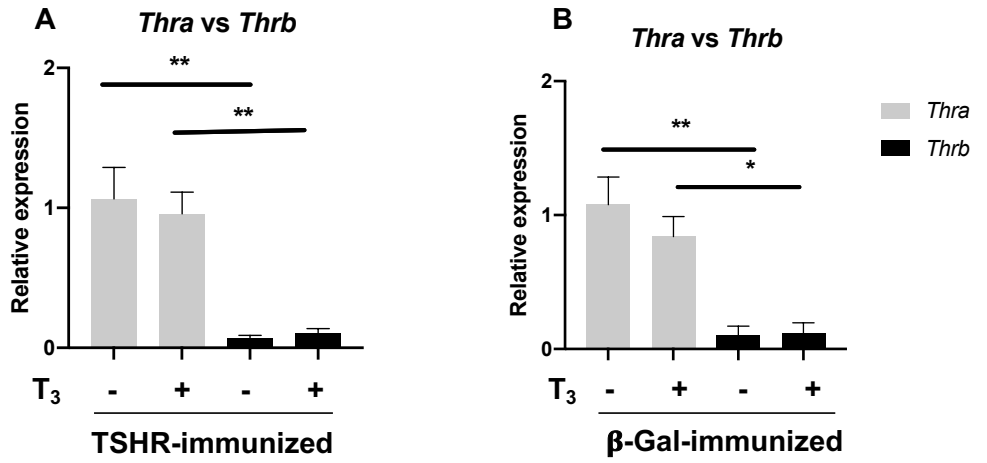

Figure 13. Comparison between *Thra* and *Thrb* gene expression in (A) TSHR-immunized mOFs and (B) *β*-Gal mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of *Thra* or *Thrb* gene expression ± SEM, relative to *Thra* expression in untreated TSHR-immunized mOFs or *Thra* expression in untreated *β*-Gal mOFs. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test. **$P < 0.005$, *$P < 0.05$).

3.2.2. T_3 treatment did not significantly induce *Klf9* gene expression in TSHR mOFs

Previously, T_3 treatment was found to significantly induce *KLF9* gene in GO OFs (Figure 10). In this experiment, the effect of T_3 treatment on *Klf9* gene expression was determined in TSHR mOFs and *β*-Gal mOFs. According to the RT-qPCR analysis, T_3 treatment

appeared to induced *Klf9* gene expression, although not statistically significant, in both mOFs (Figure 17).

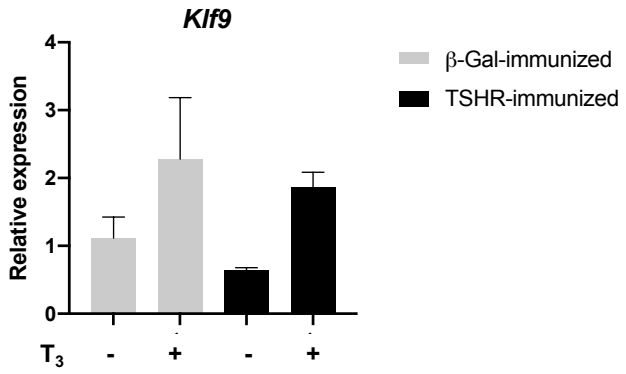

Figure 14. *Klf9* gene expression in TSHR-immunized or *β*-Gal mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated *β*-Gal mOFs. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test).

3.2.3. T_3 treatment upregulated *hr* gene expression in TSHR mOFs

hr is another known TH target gene. Its expression has been identified to be directly upregulated by T_3 in rat brain (Thompson, 1996). In this experiment, *hr* gene expression was measured to determine if T_3 mediates this gene expression in TSHR mOFs.

According to the RT-qPCR analysis, *hr* gene expression was strongly induced by T_3 treatment in TSHR mOFs (Figure 15).

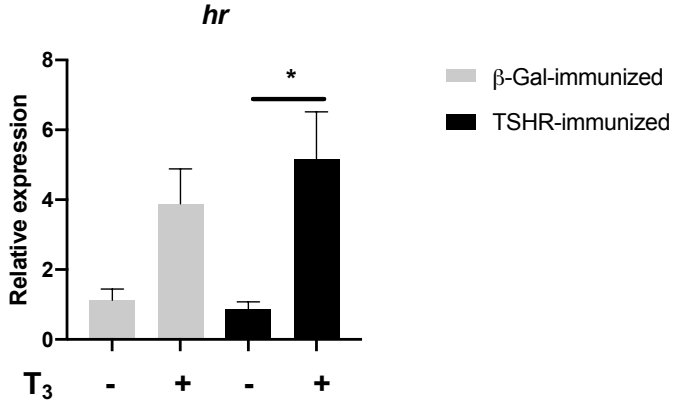

Figure 15. *hr* gene expression in TSHR-immunized or *β*-Gal mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of

every group ± SEM, relative to untreated β-Gal mOFs. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test; *$P < 0.05$).

3.2.4. *Mct8* gene expression was upregulated in TSHR mOFs

Mct8 gene expression was examined in this experiment to determine whether TSHR immunization and/or T_3 treatment affects TH transport in mOFs.

According to the RT-qPCR analysis, *Mct8* gene was significantly upregulated in TSHR-mOFs. However, T_3 treatment did not alter *Mct8* gene expression in both mOFs (Figure 16).

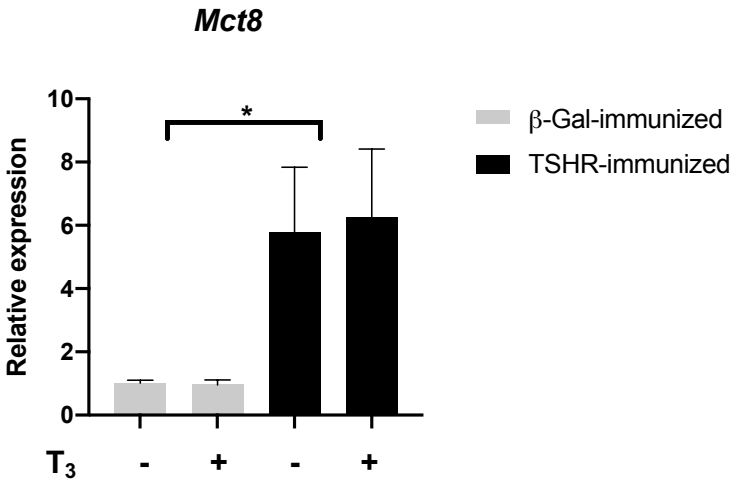

Figure 16. *Mct8* gene expression in TSHR-immunized or β-Gal mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated β-Gal mOFs. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test; *$P < 0.05$).

3.3. Evaluation of thyroid hormone responsive genes in *Thra*$^{0/0}$ mOFs

Thra was found to be predominantly expressed in GO OFs as well as in TSHR mOFs. In these series of experiments, firstly *Thra* and *Thrb* gene expression was observed and compared in WT mOFs. Subsequently, the expression of TH-responsive genes was examined in *Thra*$^{0/0}$ mOFs to observe if the high level of *Thra* gene correlates with its roles in mediating TH signalling in TSHR mOFs. *Thra*$^{0/0}$ mOFs used in these experiments were derived from C57BL/6 mice that are deficient of all known *Thra* isoforms (Gauthier et al., 2001).

3.3.1. *Thra* was predominantly expressed in WT mOFs

To determine the predominantly expressed *Thr* isoforms in WT mOFs, *Thra* and *Thrb* relative gene expression was compared based on their C*q* values. In accordance to the comparison performed in GO OFs and immunized TSHR mOFs, *Thra* gene was also predominantly expressed in WT mOFs (Figure 17).

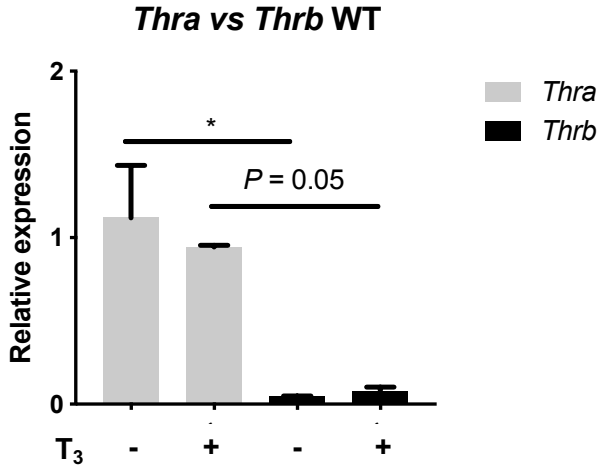

Figure 17. Comparison between *Thra* and *Thrb* gene expression in WT mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to *Thra* gene expression in untreated WT mOFs. All data were normalized to *Ppia* (n = 2; Statistical test: One-way ANOVA and Tukey's post-hoc test. *$P < 0.05$).

3.3.2. *Thrb* gene was not responsive to T_3 treatment in $Thra^{0/0}$ mOFs

The first experiment was performed to confirm lack of *Thra* gene expression in $Thra^{0/0}$ mOFs. As expected, *Thra* gene expression was absent in $Thra^{0/0}$ mOFs (Figure 18, Panel A).

In the second experiment, *Thrb* gene expression was measured in $Thra^{0/0}$ mOFs to observe if *Thrb* expression was dependent on *Thra*. According to RT-qPCR analysis, *Thrb* gene expression was not responsive to T_3 treatment in WT or $Thra^{0/0}$ mOFs (Figure 18, Panel B).

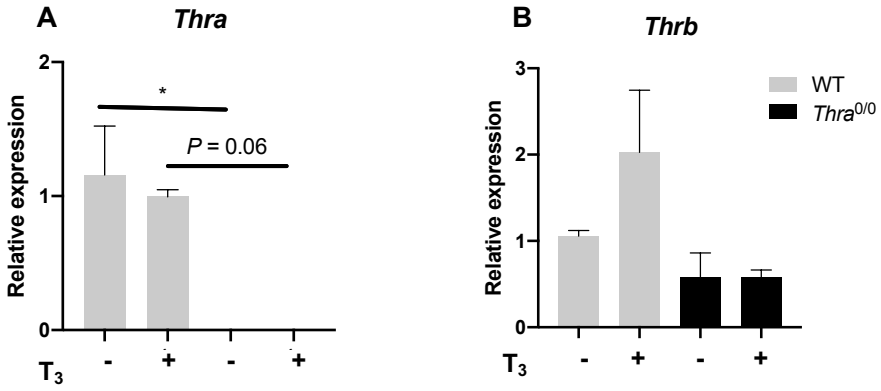

Figure 18. *Thra* and *Thrb* gene expression in *Thra*$^{0/0}$ or WT mOFs. (A) *Thra* and (B) *Thrb* gene expression in *Thra*$^{0/0}$ or WT with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT. All data were normalized to *Ppia* (n = 2; Statistical test: One-way ANOVA and Tukey's post-hoc test. *$P < 0.05$)

3.3.3. *Klf9* gene was not responsive to T_3 treatment in *Thra*$^{0/0}$ mOFs

In this experiment, *Klf9* gene expression was measured in *Thra*$^{0/0}$ to determine if *Thra* gene mediates T_3 action on *Klf9* gene in mOFs.

According to the RT-qPCR analysis, *Klf9* gene was less expressed in untreated *Thra*$^{0/0}$ mOFs compared to WT mOFs. However, the difference was not statistically significant. In addition, *Klf9* gene was not responsive to T_3 treatment in *Thra*$^{0/0}$ mOFs (Figure 19).

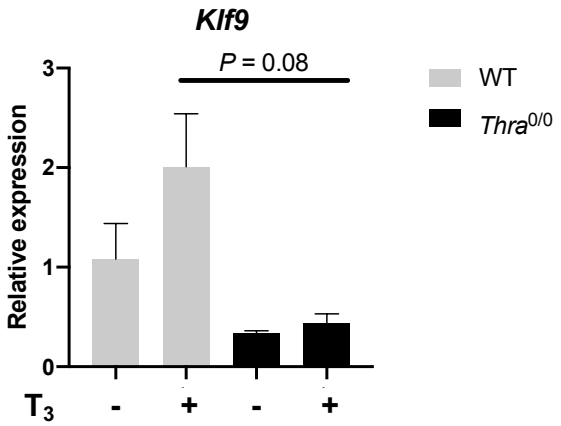

Figure 19. *Klf9* gene expression in *Thra*$^{0/0}$ or WT mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT mOFs. All data were normalized to *Ppia* (n = 2; Statistical test: One-way ANOVA and Tukey's post-hoc test).

3.3.4. *hr* gene was not responsive to T_3 treatment in *Thra*$^{0/0}$ mOFs

In this experiment *hr* gene expression was measured in *Thra*$^{0/0}$ mOFs to determine if *Thra* gene mediates T_3 action on *hr* gene in mOFs.

According to the RT-qPCR analysis, T_3 treatment induced *hr* gene expression in WT mOFs. However, this effect was absent in *Thra*$^{0/0}$ mOFs (Figure 20).

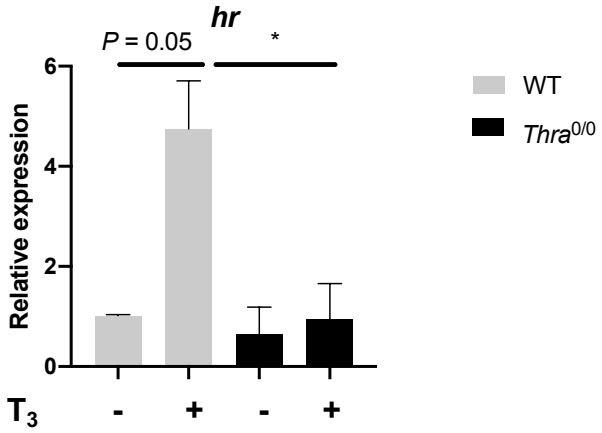

Figure 20. *hr* gene expression in *Thra*$^{0/0}$ or WT mOFs with or without T_3 treatment to the culture. Data are presented as mean ± SEM relative to untreated WT mOFs. All data were normalized to *Ppia* (n = 2; Statistical test: One-way ANOVA and Tukey's post-hoc test. *$P < 0.05$).

3.3.5. *Mct8* gene expression in *Thra*$^{0/0}$ mOFs was comparable to WT mOFs

Mct8 gene expression was significantly upregulated in TSHR mOFs (Figure 16). In this experiment, the gene expression was observed in *Thra*$^{0/0}$ mOFs to observe if *Thra* gene regulates *Mct8* gene expression. According to the RT-qPCR analysis, the absence of *Thra* gene did not affect *Mct8* gene expression in mOFs (Figure 21).

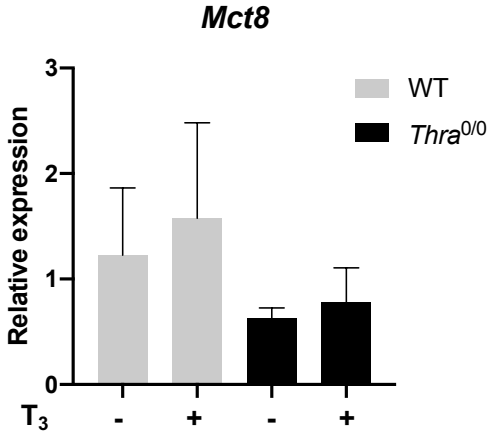

Figure 21. *Mct8* gene expression in *Thra*$^{0/0}$ or WT mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT mOFs. All data were normalized to *Ppia* (n = 2; Statistical test: One-way ANOVA and Tukey's post-hoc test.

3.4. Evaluation of thyroid hormone responsive genes in *Thrb*$^{-/-}$ mOFs

To understand the roles of *Thrb* gene in mediating TH action in mOFs, a series of experiments were performed in *Thrb*$^{-/-}$ mOFs. These mOFs were derived from C57BL/6 mice that are deficient of all known *Thrb* isoforms (Forrest et al., 1996). WT mOFs were used as controls for these experiments. The same TH-responsive genes were examined in these mOFs.

3.4.1. *Thra* gene was less expressed in *Thrb*$^{-/-}$ mOFs than WT mOFs

To confirm the absence of *Thrb* gene in *Thrb*$^{-/-}$ mOFs, firstly the gene expression was examined by RT-qPCR and compared to the expression in WT mOFs. As predicted, *Thrb* gene expression was absent in *Thrb*$^{-/-}$ mOFs, confirming lack of *Thrb* gene expression in *Thrb*$^{-/-}$ mice (Figure 22, Panel A).

In the second experiment, *Thra* gene expression was measured in *Thrb*$^{-/-}$ mOFs. Interestingly, *Thra* gene expression was significantly downregulated in *Thrb*$^{-/-}$ mOFs, compared to WT mOFs (Figure 22, Panel B).

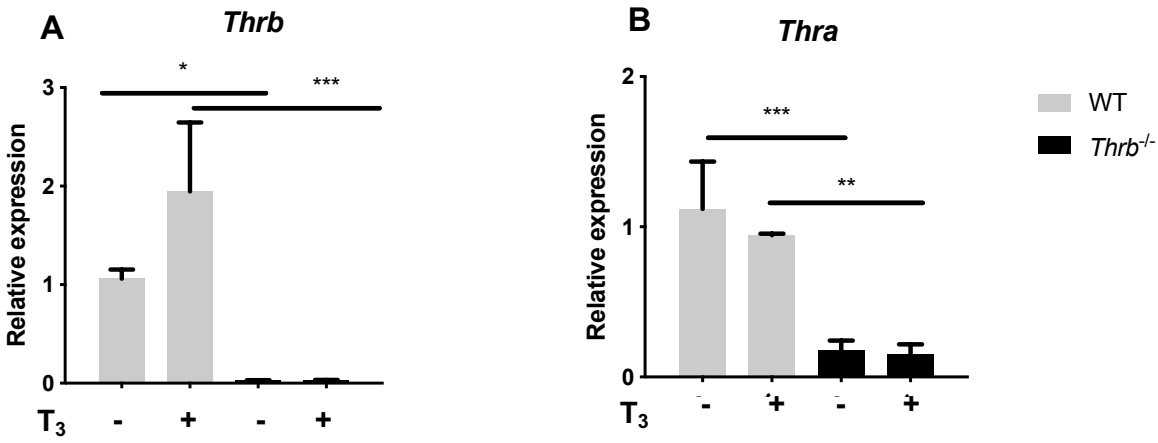

Figure 22. *Thra and Thrb* gene expression in *Thrb*$^{-/-}$ or WT mOFs. (A) *Thrb* gene expression with and without 100 nM T_3 treatment to *Thrb*$^{-/-}$ or WT mOFs. (B) *Thra* gene expression with and without 100 nM T_3 treatment to *Thrb*$^{-/-}$ or WT mOFs. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT mOFs. All data were normalized to *Ppia* (n = 2 for WT and n = 5 for *Thrb*$^{-/-}$; Statistical test: One-way ANOVA and Tukey's post-hoc test. ******P* <0.001; *****P* **< 0.005; ****P*** **< 0.05).**

3.4.2. *Klf9* gene was not responsive to T_3 treatment in *Thrb*$^{-/-}$ mOFs

Klf9 gene expression was measured in *Thrb*$^{-/-}$ mOFs to determine if *Thrb* gene mediates TH signalling in mOFs.

According to RT-qPCR analysis, *Klf9* gene was slightly less expressed in untreated *Thrb*$^{-/-}$ mOFs compared to WT mOFs. In addition, *Klf9* gene expression was not responsive to T_3 treatment in *Thrb*$^{-/-}$ mOFs (Figure 23).

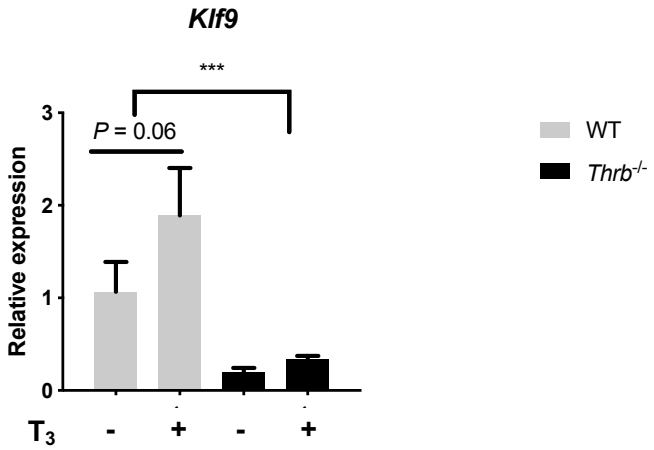

Figure 23. *Klf9* gene expression in *Thrb*$^{-/-}$ or WT mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT mOFs. All data were normalized to *Ppia* (n = 2 for WT and n = 5 for *Thrb*$^{-/-}$; Statistical test: One-way ANOVA and Tukey's post-hoc test. * *P* <0.001).**

3.4.3 *hr* gene was not responsive to T_3 treatment in *Thrb*$^{-/-}$ mOFs

The expression of *hr* gene was observed to determine if this gene expression is mediated by *Thrb* gene in mOFs. According to RT-qPCR analysis, *hr* gene expression was depleted in untreated *Thrb*$^{-/-}$ mOFs. Furthermore, T_3 treatment did not significantly induce *hr* gene expression in *Thrb*$^{-/-}$ mOFs (Figure 24).

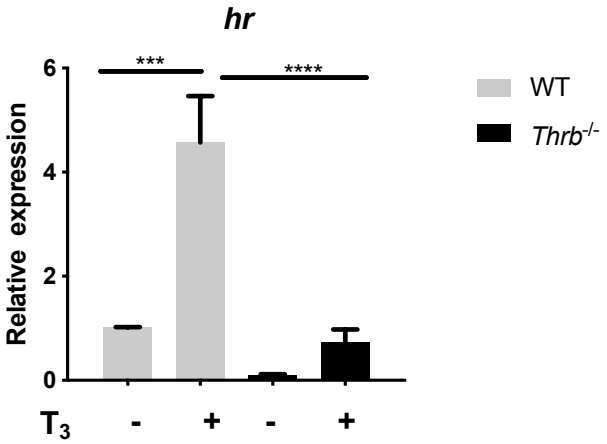

Figure 24. *hr* gene expression in *Thrb*$^{-/-}$ or WT mOFs with and without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT mOFs. All data were normalized to *Ppia* (n =

2 for WT and n = 5 for *Thrb*$^{-/-}$; Statistical test: One-way ANOVA and Tukey's post-hoc test. ** *P* < 0.0001 *** *P* < 0.001).**

3.4.2. *Mct8* gene expression in *Thrb*$^{-/-}$ mOFs is comparable to WT mOFs

In this experiment *Mct8* gene expression was examined in *Thrb*$^{-/-}$ mOFs to observe if *Thrb* regulates the gene expression. According to RT-qPCR analysis, *Mct8* gene expression in mOFs was not affected by the absence of *Thrb* gene. Furthermore, *Mct8* gene expression was not responsive to T_3 treatment (Figure 25).

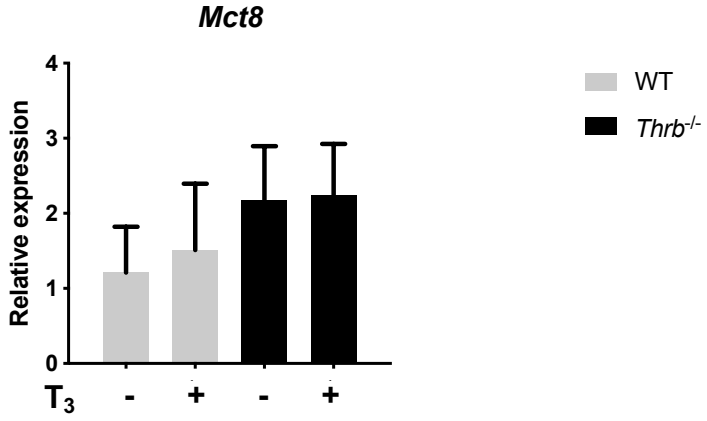

Figure 25. *Mct8* gene expression in *Thrb*$^{-/-}$ or WT mOFs with or without 100 nM T_3 treatment to the culture. Data are presented as mean of the gene expression of every group ± SEM, relative to untreated WT mOFs. All data were normalized to *Ppia* (n = 2 for WT and n = 5 for *Thrb*$^{-/-}$. Statistical test: One-way ANOVA and Tukey's post-hoc test.)

3.5. The effect of T_3 treatment in male WT C57BL/6 mice

In the previous experiments, experimental hyperthyroid was induced locally in mOFs cultures. In these experiments the effects of systemic T_3 induction in mouse orbital tissue were determined. The mice were divided into three groups, namely MMI-treated; long-term T_3-treated; and short-term T_3-treated. All mice were given drinking water enriched with MMI during the first three weeks of the experiment to render them hypothyroid. Body weight was being measured before the start of the experiment, three weeks after MMI treatment, and at the end of the experiment. Blood was withdrawn from all mice after the sacrifice. Liver and orbital tissue were isolated and used for RT-qPCR analysis.

Finally, the expression of TH responsive genes was examined in the mRNA derived from liver and orbital tissue samples.

3.5.1. MMI treatment reduced mice body weight

All mice were experiencing significant body weight reduction during the initial 3-weeks MMI treatment. There were no significant changes in their body weight between week 3 and week 4 of the experiment, regardless the treatment (Figure 26).

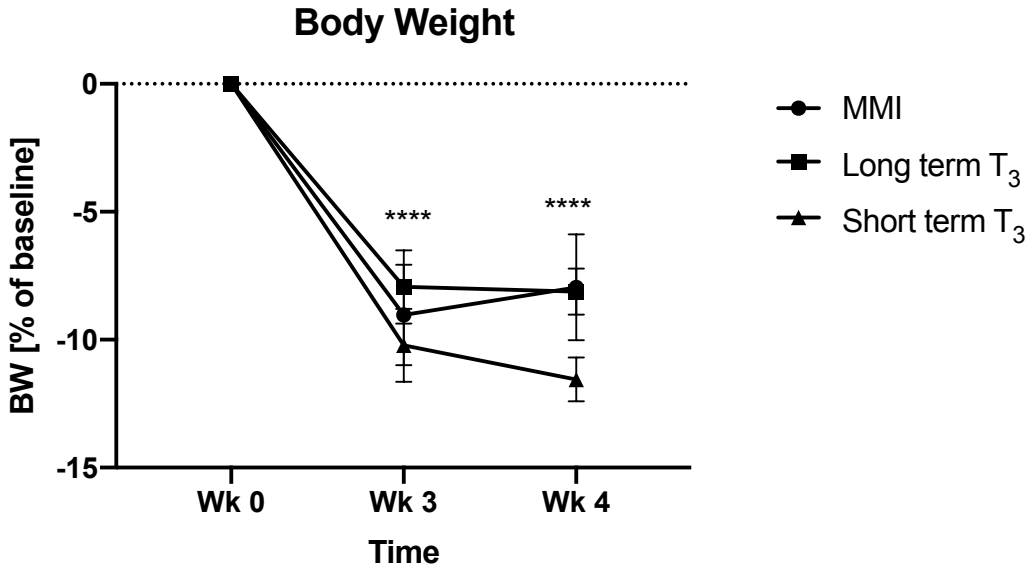

Figure 26. Changes of male WT C57BL/6 mice body weight before and after long term or short term T_3 treatment. Data are presented as mean of percent body weight change compared to baseline (Wk 0) (n = 4; Statistical test: 2-way ANOVA and Sidak's multiple comparison test; ****$P < 0.0001$; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice).

3.5.2. T_3 treatment upregulated serum FT_3 level in mice

ELISA assays were performed to measure serum free T_3 (FT_3) levels in all mice. FT_3 level was depleted in the MMI group. The long term T_3 group showed a 10-fold higher

serum FT_3 level compared to the MMI group, while the short term T_3 group showed nearly 30-fold induction in FT_3 level compared to MMI group (Figure 27).

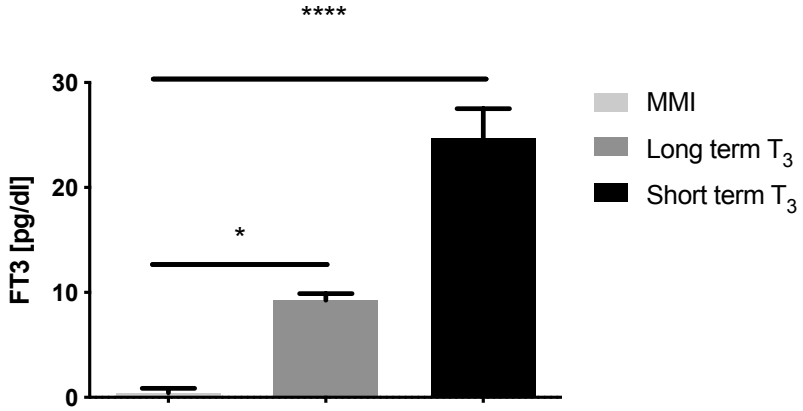

Figure 27. FT_3 level in the serum of male WT C57BL/6 mice following MMI, long term T_3, or short term T_3 treatment. All mice were rendered hypothyroid by administering MMI water before T_3 treatment. Data are presented as mean ± SEM (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test. ****$P < 0.0001$; *$P < 0.05$; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice).

3.5.3. Short term T_3 treatment induced serum total T_4 (TT_4) level in mice

ELISA assays were conducted to examine TT_4 level in all mice serum. As expected, the TT_4 level was similar between MMI group and long term T_3 group. However, TT_4 level was higher in the short term T_3 group compared to the other groups (Figure 28).

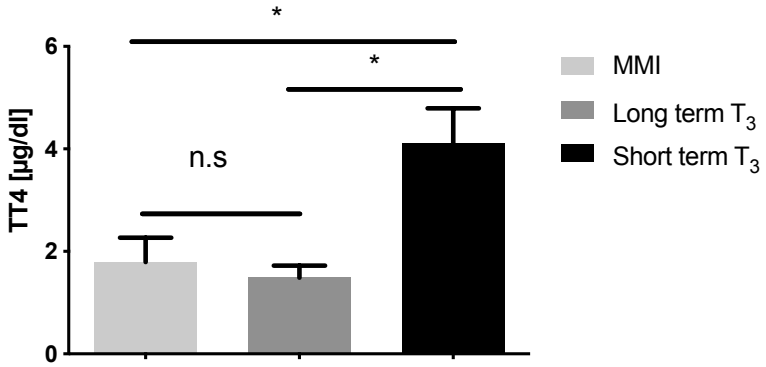

Figure 28. TT_4 level in the serum of male WT C57BL/6 mice following MMI, long term T_3, or short term T_3 treatment. All mice were rendered hypothyroid by administering MMI before T_3 treatment. Data are presented as mean ± SEM (n = 4; Statistical test: One-way ANOVA and Tukey's post-hoc test. *$P < 0.05$ n.s. = not significant; Long term

T_3 = 0.5 μg/mL T_3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice)

3.6. Evaluation of TH-responsive genes expression in male WT C57BL/6 mouse orbital tissue

The expression of TH-responsive genes was examined in orbital mRNA derived from WT mice undergoing long term T_3 treatment, to observe the effect of *in vivo* T_3 treatment on TH signalling in orbital tissues.

3.6.1. *Thra* was predominantly expressed in WT mouse orbital tissue

First, the expression of both *Thr* genes isoform was examined in MMI and long term T_3-treated mouse orbital mRNA to confirm the results obtained from previous *in vitro* experiments. Both *Thra* and *Thrb* gene expression in mouse orbital mRNA were not affected by long term T_3 treatment (Figure 29). Furthermore, *Thra* showed predominant expression in these samples (Figure 30).

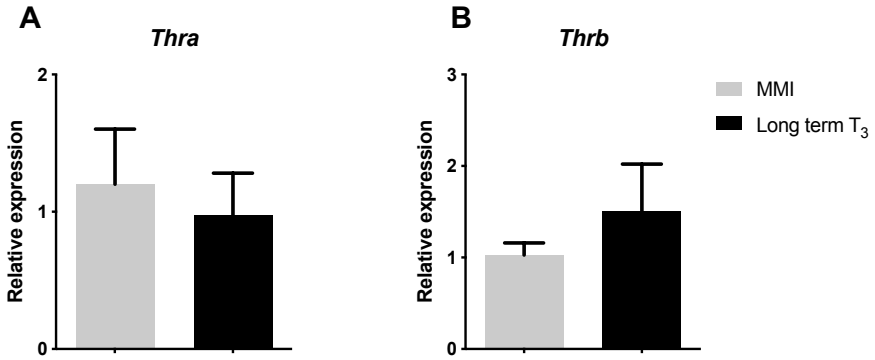

Figure 29. (A) *Thra* and (B) *Thrb* gene expression in male WT C57BL/6 mouse orbital mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were normalized to *Ppia* (n = 3-4; Statistical test: Unpaired t-test with Welch's correction; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days).

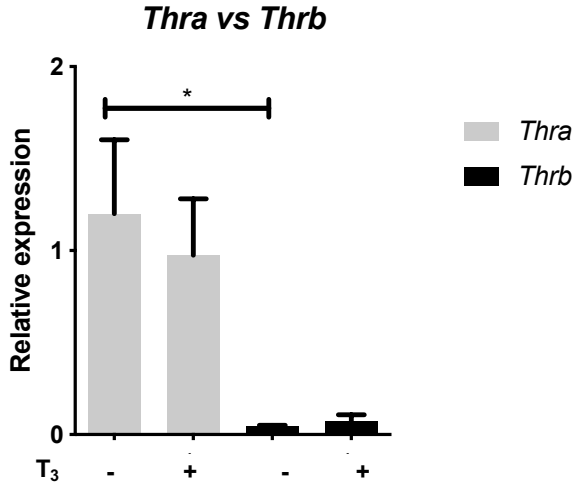

Figure 30. Comparison between *Thra* and *Thrb* gene expression in male WT C57BL/6 mouse orbital mRNA, treated with MMI (-) or T_3 for 7 days (+). Data are presented as mean of the gene expression ± SEM relative to *Thra* gene expression in MMI group. All data were normalized to *Ppia* (n = 3-4; Statistical test: One-way ANOVA, followed by Tukey's post-hoc test; *P <0.05).

3.6.2. Long term T_3 treatment upregulated *Klf9* gene expression in mouse orbital tissue

The expression of *Klf9* was observed in WT mouse orbital mRNA to observe whether *in vivo* T_3 treatment could significantly induced this gene expression. Here, according to RT-qPCR analysis, *Klf9* gene expression in orbital mRNA was significantly upregulated following a long term T_3 treatment in WT mice (Figure 31).

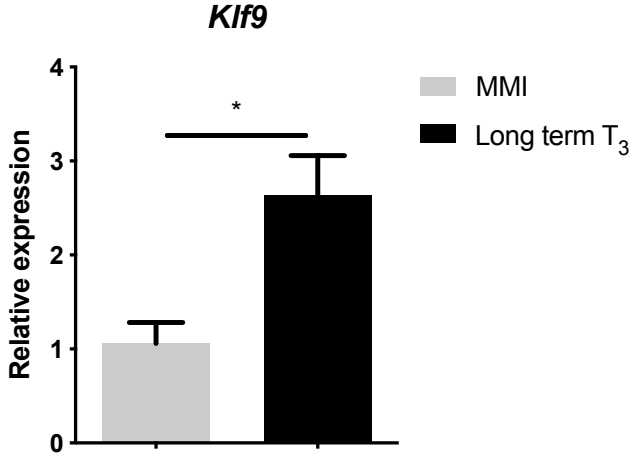

Figure 31. *Klf9* gene expression in male WT C57BL/6 mouse orbital mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were

normalized to *Ppia* (n = 3-4; Statistical test: Unpaired t-test with Welch's correction; *$P < 0.05$; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days).

3.6.3. Long term T_3 treatment did not affect *hr* gene expression in mouse orbital tissue

In this experiment, *hr* gene expression was examined in orbital mRNA derived from WT mice undergoing long term T_3 treatment to determine if the effect of T_3 on *hr* gene expression was conserved *in vivo*. According to RT-qPCR analysis, *hr* gene expression in WT mouse orbital mRNA was not significantly altered following long term T_3 treatment (Figure 32).

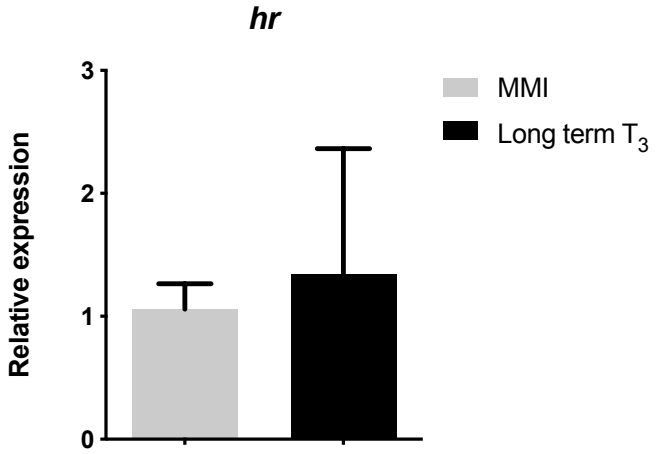

Figure 32. *hr* gene expression in male WT C57BL/6 mouse orbital mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were normalized to *Ppia* (n = 3-4; Statistical test: Unpaired t-test with Welch's correction; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days).

3.6.4. Long term T_3 treatment did not affect *Mct8* gene expression in mouse orbital tissue

According to the results of the previous *in vitro* experiments, *Mct8* gene expression was not affected by T_3 treatment in mOFs. In this experiment, *Mct8* gene expression was examined to determine if systemic T_3 induction affects its expression in mouse orbital mRNA. Similarly, RT-qPCR analysis showed no changes in *Mct8* gene expression in mouse orbital mRNA, following long term T_3 treatment (Figure 36).

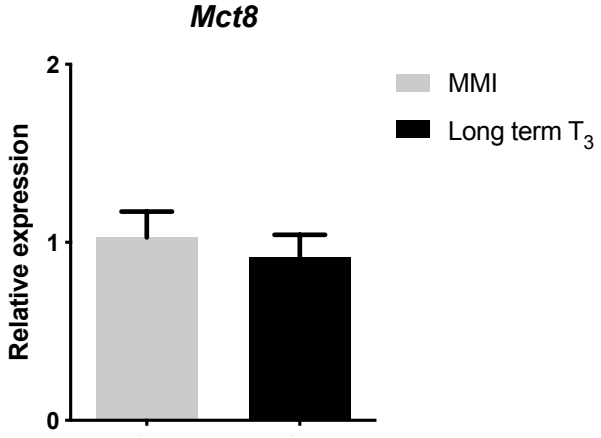

Figure 33. *Mct8* gene expression in male WT C57BL/6 mouse orbital mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were normalized to *Ppia* (n = 3-4; Statistical test: Unpaired t-test with Welch's correction; Long term T_3 = 0.5 μg/mL T3 in MMI water for 7 days).

3.7. Evaluation of TH-responsive genes in WT mouse liver

Liver is widely known as the organ that is strongly affected by T_3 induction in mammals (Chi et al., 2019). In this experiment, the expression of TH-responsive genes was examined in mouse liver mRNA as the control to determine T_3 treatment efficiency in WT mice.

3.7.1. Long term T_3 treatment downregulated *Thra* gene expression in mouse liver

According to RT-qPCR, *Thra* gene expression was significantly downregulated in liver mRNA from WT mice undergoing long term T_3 treatment (Figure 34, Panel A). Whereas, *Thrb* gene expression in mouse liver mRNA was not affected by long term T_3 treatment. However, *Thrb* gene was downregulated following short term T_3 treatment in comparison to MMI group (Figure 34, Panel B).

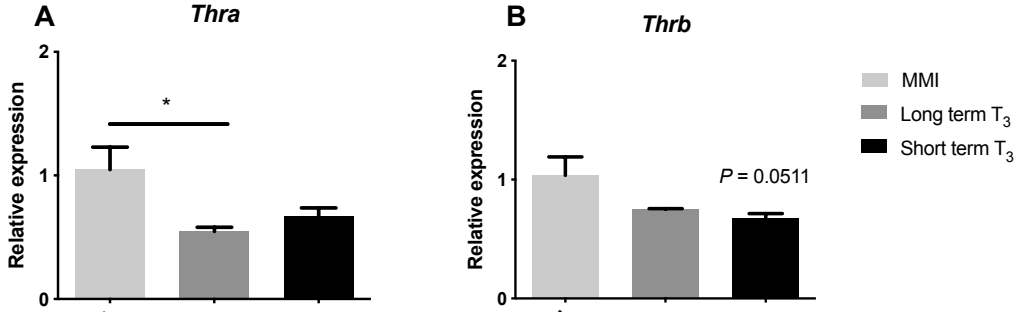

Figure 34. (A) *Thra* and (B) *Thrb* gene expression in male WT C57BL/6 mouse liver mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA followed and Tukey's post-hoc test; *$P < 0.05$; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice).

3.7.2. Long term T_3 treatment upregulated *Klf9* gene expression in mouse liver

According to RT-qPCR analysis, *Klf9* gene expression was significantly upregulated in liver mRNA derived from WT mice undergoing long term T_3 treatment. The gene expression was not altered in the short term T_3 group (Figure 35).

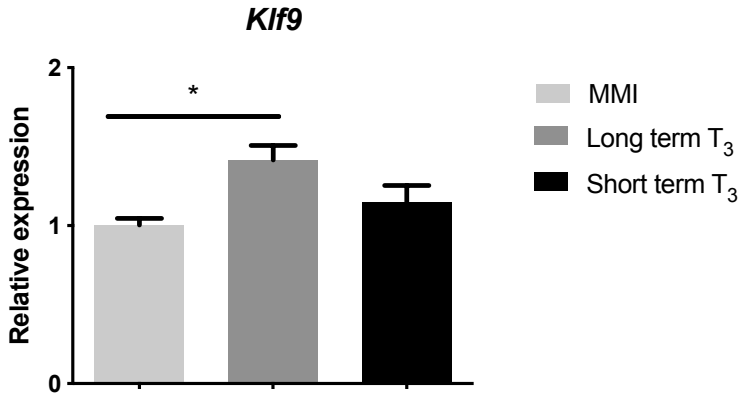

Figure 35. *Klf9* gene expression in male WT C57BL/6 mouse liver mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA followed and Tukey's post-hoc test; *$P < 0.05$; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice).

3.7.3. Long term T_3 treatment upregulated *hr* gene expression in mouse liver

According to the RT-qPCR analysis, *hr* gene expression was significantly upregulated in the liver mRNA derived from WT mice undergoing long term T_3 treatment. The expression of *hr* gene in short term T_3 group was similar to the MMI group (Figure 36).

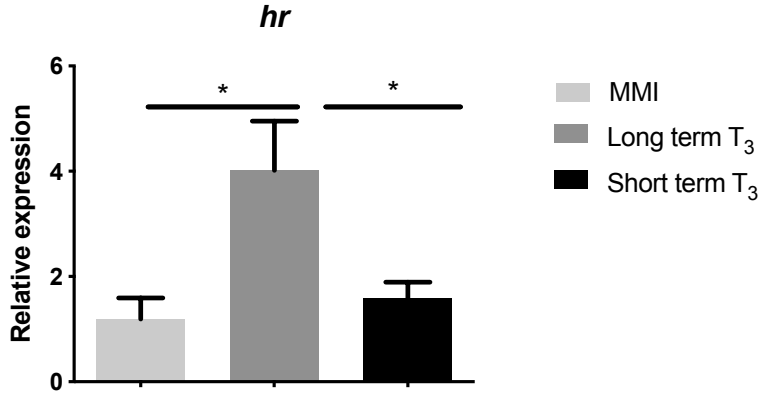

Figure 36. *hr* gene expression in male WT C57BL/6 mouse liver mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA followed and Tukey's post-hoc test; *$P < 0.05$; Long term T_3 = 0.5 μg/mL T_3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice).

3.7.2. Short term T_3 treatment upregulated *Mct8* gene expression in mouse liver

According to the RT-qPCR analysis, *Mct8* gene expression was not changed in liver mRNA following long term T_3 treatment. Interestingly, *Mct8* expression in liver mRNA showed a significant upregulation in the short term T_3 group (Figure 37).

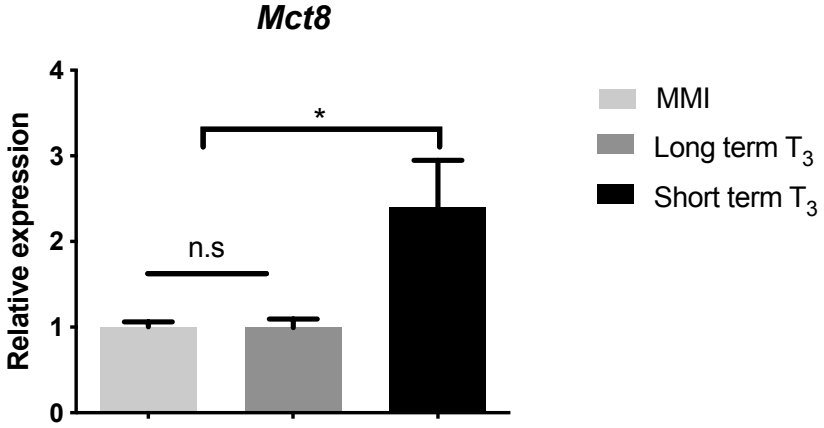

Figure 37. *Mct8* gene expression in male WT C57BL/6 mouse liver mRNA. Data are presented as mean of the gene expression ± SEM relative to MMI group. All data were

normalized to *Ppia* (n = 4; Statistical test: One-way ANOVA followed and Tukey's post-hoc test; *$P < 0.05$; Long term T_3 = 0.5 μg/mL T3 in MMI water for 7 days; Short term T_3 = 20 μg/100 g body weight T_3 i.p injection 6 hours before sacrifice).

CHAPTER 4: DISCUSSION

4.1. The expression of *THRA* and *THRB* in GO OFs

This is the first study to examine *THR* gene expression in GO OFs. The initial experiments performed in this thesis showed the expression of *THR* isoform genes (*THRA* and *THRB*) in GO OFs, with the predominant expression of *THRA*. Similarly, predominant expression of *THRA* was previously detected in multipotent human adipocyte stem cells (hADSC) (Cvoro et al., 2016), human red cell progenitors (Gamper et al., 2009), skeletal muscle, the heart, and pancreas (Shahrara et al., 1999).

Furthermore, both *THRA* and *THRB* gene expression in GO OFs were not responsive to T_3 treatment. In contrast, *THRB* was significantly upregulated by T_3 treatment in control OFs. Previously, Cvoro and colleagues reported that both *THRA* and *THRB* were not responsive to T_3 in preadipocyte-derived hADSC. However, both *THRA* and *THRB* were responsive to T_3 in differentiated adipocyte-derived hADSC cells (Cvoro et al., 2016). In the present study, GO OFs cultures were not subjected to adipocyte differentiation. This might be the reason why *THRA* and *THRB* were not responsive to T_3 treatment in GO OFs. The T_3-induced *THRB* upregulation in control OFs can be explained by the presence of TRE in the promotor region of human *THRB*, as described by Sakurai and colleagues in *THRB* isolated from human placenta genomic library (Sakurai et al., 1992). In that study, they proposed that the TRE present in human *THRB* gene promoter might involve in the autoregulation of human *THRB* gene expression (Sakurai et al., 1992).

Taken together, both *THR* genes were expressed in GO OFs with *THRA* being the predominantly expressed receptor. Furthermore, T_3 treatment did not significantly affect both *THR* expression in GO OFs. This phenomenon might be due to the generally low expression of both *THR* gene in non-differentiated GO OFs. Furthermore, since *THRB* gene was less responsive to T_3 treatment in GO OFs compared to control OFs, GO condition might reduce the interaction between T_3 and *THRB*.

4.2. The expression of *Thra* and *Thrb* in TSHR mOFs

Following the study of *THR* genes expression in human OFs, *Thr* genes were examined in mouse TSHR OFs (TSHR mOFs). These mOFs were derived from a preclinical GO mouse model, first described by Moskelgosha and colleagues in 2013 (Moshkelgosha et

al., 2013) and now established in our laboratory (Berchner-Pfannschmidt et al., 2016, Plohn et al., 2019, Schluter et al., 2018).

In the present study, *Thra* and *Thrb* genes were expressed in TSHR mOFs. In accordance to their expression in GO OFs, *Thra* was predominantly expressed in TSHR mOFs. Furthermore, *Thra* and *Thrb* gene expression was not responsive to T_3 treatment.

Our group has reported that TSHR mOFs are rich in MSC markers, such as CD40.2 and CD90 (Görtz et al., 2016b). Furthermore, an earlier study described *Thra* predominant expression in mouse neural stem cells (Lemkine et al., 2005). Thus, the predominant expression of *Thra* in TSHR mOFs might also indicate their MSC properties.

A previous study on Ob 17 rat preadipocyte cell lines showed that *Thra* gene is always detected at high levels in preadipocytes and adipocytes (Dace et al., 1999). Whereas, *Thrb* gene was detected at low levels in the early preadipocytes but abolished in late preadipocytes and maturing adipocytes. Treatment with 1.5 nM T_3 restored *Thrb* gene expression in maturing adipocytes (Dace et al., 1999). This indicated that *Thra* gene, but not *Thrb* gene, expression in murine Ob 17 preadipocyte cell lines is T_3-independent. A similar effect was found in rat BAT cells (Hernández and Obregón, 1996).

Thra gene expression in TSHR mOFs as well as in control β-Gal might also be T_3-independent, since it was not responsive to T_3 treatment. On the other hand, unlike in mouse preadipocytes, *Thrb* gene in mOFs might not be sensitive to T_3 treatment, as the effect of T_3 on *Thrb* has been known to be tissue-specific (Hodin et al., 1990).

Additional supporting evidence on the expression of *Thra* and *Thrb* gene in TSHR mOFs would be expression at the protein level. However, examination of Thra and Thrb protein is currently difficult because there are no reliable antibodies against Thr. A recent study conducted in mouse models expressing hemagglutinin (HA)-tagged Thr to monitor the protein expression of Thr reported the predominant expression of Thra in all peripheral organs except in the liver (Minakhina et al., 2020). The investigators confirmed similar expression of *Thra* on mRNA level. In contrast, Thra protein level in fat and muscle tissues were found to be 10 times higher than Thrb protein level, though their mRNA levels were comparable. This shows that enumeration of *Thr* gene expression at the mRNA level does not strictly correlate with protein expression levels. They concluded that Thra and Thrb expression is regulated both at the transcriptional and post transcriptional level and organ-specific manner (Minakhina et al., 2020). Therefore, even

though *Thra* gene is predominantly expressed in TSHR mOFs, the expression of Thra and Thrb protein might be different in these mOFs.

4.3. The expression of *KLF9* gene in GO OFs

The next question addressed in this thesis was whether *THRA* and *THRB* gene in GO OFs mediate T_3 actions. Therefore, the expression of a known TH responsive gene, *KLF9*, was examined in GO OFs. *KLF9* gene expression in GO OFs was upregulated following overnight T_3 treatment.

The interaction between T_3 and *KLF9* is often associated with developmental processes in human cells (Cvoro et al., 2016, Cvoro et al., 2015). It was reported that *KLF9* gene expression is activated in human HepG2 cells 24 hours after T_3 treatment (Cvoro et al., 2015). T_3 was also previously reported to induce *KLF9* gene expression in differentiated human red cells progenitors (Gamper et al., 2009). In another study, Cvoro and colleagues reported that T_3 affects *KLF9* gene expression in hADSC-derived adipocytes but not in hADSC-derived preadipocytes (Cvoro et al., 2016).

Furthermore, T_3 addition into genetically-engineered HepG2 cells that highly expressed *THR* have been shown to rapidly induce *KLF9* gene expression. This finding described the interaction between T_3 and *THR* in inducing *KLF9* gene expression in HepG2 cells (Cvoro et al., 2015). This is in agreement with *KLF9* gene expression in OFs, which express both *THRA* and *THRB*. Taken together, *KLF9* is a direct T_3 target gene in OFs. Furthermore, *THRA* and *THRB* might mediate T_3 action on *KLF9* expression in OFs. However, further examinations are needed to determine if T_3-induced *KLF9* upregulation plays a role in GO development.

4.4. The expression of *Klf9* gene in TSHR mOFs

The expression of *Klf9* was also examined in TSHR mOFs. TSHR mOFs expressed *Klf9*. However, unlike in OFs, *Klf9* gene expression was only weakly induced by T_3 in mOFs. Studies on developing rat brain (Denver et al., 1999), mouse liver cells (Cvoro et al., 2015), mouse oligodendrocytes (Dugas et al., 2012), and mouse cerebrocortical cell cultures (Gil-Ibanez et al., 2014) reported induction of *Klf9* gene expression following T_3 treatment.

Denver and colleagues described T_3 regulation on *Klf9* gene as neural cell-specific (Denver et al., 1999). T_3 upregulated *Klf9* expression in primary cultures of embryonic neurons and in neonatal astrocytes, but not in neonatal oligodendrocytes (Denver et al., 1999). T_3 treatment was also reported to induce *Klf9* gene expression in concentration-dependent manner in primary hepatocytes derived from male C57BL/6 mice (Cvoro et al., 2015). Dugas and colleagues found that exposure to T_3 for 3 hours and 3 days induced *Klf9* expression in oligodendrocyte precursor cells (Dugas et al., 2012). In a study conducted by Gil-Ibanez and colleagues, it was reported that the addition of 1 nM T_3 induced *Klf9* gene expression in primary cell cultures derived from the embryonic mouse cerebral cortex (Gil-Ibanez et al., 2014).

The identification of TRE at the direct repeat 4 of *Klf9* gene transcription site in mouse brain by Denver and Williamson in 2009 supported the direct role of T_3 in regulating *Klf9* gene expression (Denver and Williamson, 2009). Thus, *Klf9* is a direct T_3 target gene. The weak induction of *Klf9* in mOFs following overnight T_3 treatment might be caused by the MSC characteristics of mOFs. It was previously reported that *KLF9* gene expression is not affected by T_3 treatment in hADSCs-derived preadipocytes, because these cells only express low level of *THR* (Cvoro et al., 2016). In the present study, mOFs were not subjected to adipocyte differentiation. Therefore, T_3 treatment could not significantly induce *Klf9* gene expression in mOFs probably due to the low level of *Thr* in the non-differentiated mOFs.

Taken together, *Klf9* might be a direct T_3 target gene in mOFs. T_3 might only be able to optimally induce *Klf9* in differentiated mOFs. However, based on the current result, the role of T_3 on *Klf9* and its contribution to GO development could not be determined.

4.5. The expression of *hr* gene in TSHR mOFs

The next TH responsive gene examined in TSHR mOFs was hairless (*hr)*. TSHR mOFs expressed *hr* gene. In addition, T_3 treatment significantly upregulated *hr* gene expression in TSHR mOFs. However, T_3 treatment to β-Gal immunized mOFs did not significantly upregulate *hr* gene expression.

Hairless *(hr)* gene encodes a protein that is known to play a role in mammalian CNS development (Thompson and Bottcher, 1997) and was first characterized as a direct T_3 target gene in neonatal rat brain (Thompson, 1996). TRE was also identified in rat *hr*

gene, which support the direct activation of this gene by T_3 (Thompson and Bottcher, 1997). Gil-Ibanez and colleagues later reported *hr* induction following T_3 treatment in the primary mouse cerebrocortical cell cultures. Furthermore, T_3 action on *hr* expression was not mediated by a specific *Thr* (Gil-Ibanez et al., 2014).

The crosstalk between mouse Hr protein and p53 has been demonstrated to downregulate p53 expression and repress pro-apoptotic mechanisms regulated by p53 in the brain (O'Driscoll and Bressler, 2010). It has also been reported that retinoic acid treatment to GO OFs upregulates p53 protein expression, while reduces cAMP production (Pasquali et al., 2003). However, in that study, the investigators did not observe the effect of retinoic acid treatment on control OFs. Therefore, it is not known if the effect is GO OF-specific. In summary, *hr* is a direct T_3 target gene in mOFs. Furthermore, the significant upregulation of *hr* following T_3 treatment in TSHR mOFs might be a marker of GO development.

4.6. The expression of *MCT8/Mct8* gene in GO OFs and TSHR mOFs

The next question addressed in this thesis was whether TH transport in GO OFs as well as TSHR mOFs is regulated by T_3. TH must be transported through the plasma membrane to exert its roles in specific target cells or tissues (Groeneweg et al., 2020). There are currently several proteins that have the capacity to transport TH across the plasma membrane, including MCTs, OATPs, and LATs (Groeneweg et al., 2020).

Among those known transporters, MCT8 has been characterized as the most specific and active TH transporter (Friesema et al., 2003). Therefore, *MCT8/Mct8* gene expression in GO OFs and TSHR mOFs was examined in the present study.

MCT8/Mct8 gene was expressed in GO OFs and in TSHR mOFs. While *Mct8* gene expression was significantly upregulated in mOFs derived from animals undergoing experimental GD/GO, *MCT8* expression in GO OFs was comparable to its expression in control OFs. Furthermore, T_3 treatment did not affect the expression of *MCT8/Mct8* in both OFs.

The available literatures on human studies are currently limited to the correlation of T_3 level with MCT8-deficient condition in patients (van Geest et al., 2021). However, a previous study in mouse thyroid gland revealed an upregulation of *Mct8* mRNA following low iodine treatment *in vivo*. The expression of *Mct8* was brought back to

normal level by T_3 treatment (Hu et al., 2014). The authors proposed that *Mct8* was upregulated in hypothyroid mice to increase TH transport from the thyroid gland to the blood (Hu et al., 2014). In contrast, Rakov and colleagues reported that *Mct8* gene in mouse liver, heart, and BAT was not altered in mice following T_4 i.p treatment in comparison to control animals (Rakov et al., 2016).

Two recent studies reported that MCT8 expressed by thyroid gland maintains intra-thyroid autoregulation and capable to sense serum TH availability (Venugopalan et al., 2021, Weber et al., 2017). Therefore, the effect of T_3 on *Mct8* might be thyroid-specific. Other possibilities are that the effect of *in vivo* T_3 treatment might be different with its *in vitro* effect on *Mct8* expression or T_4 treatment might affect *Mct8* differently compared to T_3 treatment.

In humans, inter-individual variations might play a role in affecting *MCT8* gene expression. Each individual included in the study might had been experiencing different treatment before undergoing orbital decompression surgery or had different smoking habits. The average expression of *MCT8* gene was similar in all group. However, this result might not reflect the real condition in each individual.

On the other hand, in mice, *Mct8* gene expression was higher in TSHR mOFs compared to β-Gal mOFs. This might be a consequence of TSAbs production in TSHR-immunized mice. It was previously reported by our group that hTSHR A-subunit plasmid immunization to female BALB/c mice results in the production of serum TSAbs (Berchner-Pfannschmidt et al., 2016). Another study also detected the upregulation of MCT8 protein expression in rat follicular thyroid cells, following TSH treatment. This explained the role of MCT8 in regulating TH synthesis and release from the thyroid gland (Badziong et al., 2017). Therefore, high expression of *Mct8* in TSHR mOFs could be the manifestation of TSAbs in the circulation, which triggered uncontrolled production of T_3 and T_4 by thyroid gland. Finally, high level of T_3 in the circulation resulted to more *Mct8* being expressed by mOFs to facilitate T_3 transport.

Taken together, the effect of T_3 on *MCT8/Mct8* expression might be specific to cells or tissues that produce TH, such as thyroid. Therefore, T_3 treatment to OFs did not affect *MCT8/Mct8* expression. Moreover, TSAbs production due to hTSHR A-subunit plasmid immunization in mice might induce *Mct8* expression in mOFs.

4.6. The expression of *Thr*a and *Thrb* in *Thra*$^{0/0}$ or *Thrb*$^{-/-}$ mOFs

To assess the expression of *Thr* genes in *ThrKO* mOFs, *Thra* and *Thrb* gene expression was examined in mOFs derived from *Thra*$^{0/0}$ or *Thrb*$^{-/-}$ mouse. As expected, *Thra* gene was not expressed in *Thra*$^{0/0}$ and *Thrb* was not expressed in *Thrb*$^{-/-}$ mOFs. Nonetheless, *Thrb* expression was not significantly affected in *Thra*$^{0/0}$ mOFs. Whereas, *Thra* was expressed at low levels in *Thrb*$^{-/-}$ mOFs.

An earlier study showed that *Thrb*$^{-/-}$ mice display elevated TH and TSH level, which are signs of hyperthyroidism. This explains the involvement of *Thrb* in T_3-dependent feedback regulation of TSH transcription. In that study, the authors mentioned that this effect cannot be substituted by *Thra* (Forrest et al., 1996). However, a follow-up study in *Thrb*$^{-/-}$ and WT mice showed that *Thra* can mediate TSH negative feedback regulation under hyperthyroid condition. This effect was enhanced by *Thrb* in WT mice (Weiss et al., 1997). A further study performed in mice lacking both *Thr* isoforms showed an even more severe hyperthyroidism, compared to *Thrb*$^{-/-}$ (Gauthier et al., 1999). The authors argued that *Thra* plays a role in maintaining TSH negative feedback regulation in the absence of *Thrb* (Gauthier et al., 1999).

These might explain *Thrb* expression in *Thra*$^{0/0}$ mOFs. *Thrb* alone can maintain T_3-dependent feedback regulation of TSH transcription. Therefore, in the absence of *Thra*, the expression of *Thrb* in mOFs was not significantly changed. On the other hand, feedback regulation of TSH transcription was disrupted in *Thrb*$^{-/-}$ mice, resulting in persistently high TSH level despite hyperthyroidism. *Thra* could not optimally maintain feedback regulation in the absence of *Thrb*. In the end, the extreme hyperthyroid condition might lead to *Thra* suppression in *Thrb*$^{-/-}$ mOFs.

4.7. The expression of *Klf9* and *hr* gene in *Thra*$^{0/0}$ or *Thrb*$^{-/-}$ mOFs

To confirm whether T_3 can regulate the expression of the tested TH target genes in the absence of *Thra* or *Thrb*, *Klf9* and *hr* genes were examined in *Thra*$^{0/0}$ and *Thrb*$^{-/-}$ mOFs. The results showed that *Klf9* and *hr* gene were not responsive to T_3 treatment in *Thra*$^{0/0}$ as well as in *Thrb*$^{-/-}$ mOFs.

An earlier study performed on engineered neuro-2a cell cultures that either express *Thra1* or *Thrb1* showed that T_3 only upregulates *Klf9* gene expression in the engineered cell culture that express *Thrb1* and not the one that express *Thra1* (Denver et al., 1999; Denver

and Williamson, 2009). This means that T_3 regulates *Klf9* through a specific interaction with *Thrb1* in neural cells (Denver et al., 1999, Denver and Williamson, 2009). The role of T_3/*Thr* complex on *Klf9* and *hr* gene expression was also studied in mouse cerebrocortical cell cultures (Gil-Ibanez et al., 2014). In that study, the authors showed that the effect of T_3 treatment on *Klf9* and *hr* gene expression in primary mouse cerebrocortical cell cultures was independent to a specific *Thr* (Gil-Ibanez et al., 2014). Thus, the requirement of *Thr* as a mediator of T_3 actions on *Klf9* and *hr* gene expression might be cell-specific.

Taken together, the presence of *Thra* and *Thrb* appears to be mandatory to mediate T_3 induction on *Klf9* and *hr* gene expression in mOFs.

4.8. The expression of *Mct8* in *Thra*$^{0/0}$ or *Thrb*$^{-/-}$ mOFs

To investigate if TH transport to mOFs depends on the presence of *Thra* or *Thrb*, *Mct8* gene expression was evaluated in *Thra*$^{0/0}$ as well as in *Thrb*$^{-/-}$. The result showed that *Mct8* gene expression was not significantly different in *Thra*$^{0/0}$ or *Thrb*$^{-/-}$ mOFs compared to WT mOFs. This indicates that *Mct8* gene expression in mOFs is *Thr*-independent. Thus, TH transport to mOFs appears to not require either *Thra* or *Thrb*.

4.9. The effect of *in vivo* T_3 treatment in WT C57BL/6 mice

To ensure that T_3 treatment showed the same effect *in vivo*, WT C57BL/6 mice were treated with T_3 for long term or short term periods. Briefly, mice in the long term period group were treated with T_3 through their drinking water for 7 days. Whereas, mice in the short-term period group were treated with T_3 through i.p injection 6 hours before they were being sacrificed. Before starting T_3 treatment, all mice were treated with MMI for 3 weeks to render the mice hypothyroid. MMI treatment for 3 weeks significantly reduced mice body weight. The addition of T_3 on day 22 did not significantly change the body weight until mice were sacrificed on day 28. While there have been lack of studies on the correlation between MMI treatment and body weight changes in mice, a previous study showed body weight reduction in rats following MMI treatment (Herwig et al., 2014). Rats treated with MMI showed lower body weight in comparison to control rats from day 3 of the experiment and became significantly different from day 11 onwards.

Furthermore, the addition of T_3 on day 22 slightly decreased rat body weight. This indicates that hypothyroidism disrupts rat growth (Herwig et al., 2014).

Serum FT_3 levels was not detected in MMI-treated mice. Long term T_3 treatment increased serum FT_3 levels for 10-fold. Moreover, short term T_3 treatment induced serum FT_3 levels for almost 30-fold compared to MMI group. Herwig and colleagues described that serum T_3 levels were reduced by 50% in MMI-treated rats. Furthermore, T_3 administration via i.p. injection to MMI-treated rats increased serum T_3 levels by 30-fold (Herwig et al., 2014). In the present study, there were no control group due to the mice availability. Therefore, the effect of MMI treatment on FT_3 levels could not be compared to an untreated control group.

Serum TT_4 was detected in MMI-treated mice. Long term T_3 treatment did not significantly reduce serum TT_4 levels further compared to MMI treatment. However, short term T_3 treatment significantly increased serum TT_4 levels, possibly due to replacement of T_4 at serum TH binding proteins such as TBG and Albumin.

4.10. The effect of long term T_3 treatment on *Klf9* expression in WT mouse orbital mRNA

Long term T_3 treatment resulted in significant upregulation of *Klf9* gene expression in mouse orbital mRNA. This was different to the phenomenon observed in WT mOFs. T_3 treatment did not significantly induce *Klf9* gene in WT mOFs. This suggests that T_3 might need longer time to induce *Klf9* gene in mouse orbital tissues. Another possible reason is that the effect of *in vivo* T_3 treatment might be stronger than *in vitro* T_3 treatment on mouse orbital tissues.

4.11. The effect of long term T_3 treatment on *hr* expression in WT mouse orbital mRNA

Long term T_3 treatment to WT mice did not affect *hr* gene expression. This result was surprising, since *hr* gene was significantly upregulated following T_3 treatment in WT mOFs. In the study that revealed the direct role of T_3 on *hr* gene expression in rat brain, Thompson performed subcutaneous injection of T_3 to the rat for 48 hours (Thompson, 1996).

Thus, it is possible that oral administration of T_3 to the mice is not effective in inducing *hr* gene expression. Another possibility could be that long term T_3 treatment stabilizes, instead of upregulates, *hr* gene expression in mouse orbital mRNA.

4.12. The expression of *Thra* and *Thrb* in WT mouse liver mRNA

Thra expression was downregulated in the liver mRNA derived from long term T_3 treatment group, while *Thrb* expression was downregulated in short term T_3 treatment group. T_3 is known to regulate autophagy in mouse liver. This mechanism is *Thr*-dependent (Sinha et al., 2012). This might be the reason why *Thrb* was downregulated in short term T_3 treatment group, while *Thra* was downregulated in long term T_3 treatment group. Both *Thr* are needed to mediate T_3 action in mouse liver.

4.13. The expression of TH target genes in WT mouse liver mRNA

To determine if TH can exert its functions in mouse liver, the expression of *Klf9* and *hr* gene was assessed in the liver mRNA. *Klf9* expression was significantly upregulated in the liver mRNA derived from long term T_3 treatment group. This result is consistent with a previous study by Cvoro and colleagues who reported two-fold upregulation of *Klf9* gene expression in mice liver following 3 days of oral T_3 treatment, in comparison to untreated control (Cvoro et al., 2015).

Similar to *Klf9*, *hr* gene expression was also significantly upregulated in long term T_3 treatment group in comparison to MMI group. There have been no other data so far on *hr* gene expression in the liver of T_3-treated mice. However, a western blot analysis reported that Hr protein is not expressed in the liver derived from hypothyroid rats (Potter et al., 2002). Taken together, the present study showed that T_3 administration to WT mice upregulates TH target genes in mice liver mRNA. Therefore, liver is a good positive control for studying the effectivity of T_3 treatment in other mouse organs.

4.14. The expression of *Mct8* in WT mouse liver mRNA

To determine if T_3 treatment directly affect TH transport in WT mice liver, *Mct8* expression was measured. *Mct8* expression in long term T_3 treatment group was similar to the MMI group. Interestingly, *Mct8* gene expression was upregulated in short term T_3 treatment group. A previous study by Engels and colleagues showed unaltered expression

of *Mct8* in the liver of mice induced with T_4 in comparison to euthyroid mice (Engels et al., 2015). It is worth to note that the effect of T_4 treatment might be different with T_3 treatment in certain tissues. Furthermore, the effect of T_3 treatment on *Mct8* gene expression in the present study could only be compared with MMI group since euthyroid mice were not included in the study. However, a study conducted on mouse thyroid reported an increase in *Mct8* gene expression in mice treated with low iodine diet for 3 months. The expression of *Mct8* gene was decreased and returned to the same level as the control group following T_3 treatment (Hu et al., 2014). The low iodine/MMI treatment for 3 weeks in the present study might not be enough to significantly increased *Mct8* gene expression in the mouse liver. Therefore, *Mct8* gene expression did not changed after administering T_3 orally for 1 week. Lastly, the significant upregulation of *Mct8* expression in short term T_3 treatment group might be correlated to serum FT_3 and serum TT_4 induction. Taken together, T_3 treatment appears to affect *Mct8* gene expression in mouse liver.

4.15. Backcrossing

To better understand the role of *Thra* and *Thrb* in GO, a specific mouse model is needed. The mouse model should present GO phenotypes, while also lacking of *Thra* or *Thrb*. At the moment, GD and GO can only be introduced in mouse with BALB/c background (Berchner-Pfannschmidt et al., 2016, Moshkelgosha et al., 2013, Plohn et al., 2019, Schluter et al., 2018). However, the available $Thra^{0/0}$ and $Thrb^{-/-}$ mouse models are generated in C57BL/6 background (Forrest et al., 1996, Gauthier et al., 2001). Therefore, male $Thra^{+/0}$ or $Thrb^{+/-}$ C57BL/6 mice were backcrossed to female BALB/c mice for eight generation and genotyping for those mice were performed. The backcrossing experiments took two years to complete to finally attain F8 generation that was >99% BALB/c background. Furthermore, $Thra^{+/0}$ or $Thrb^{+/-}$ littermates generated from the eighth generation were inter-crossed with each other to generate $Thra^{0/0}$ or $Thrb^{-/-}$ BALB/c mice. However, the experiments on induction and progression of experimental GD/GO on these KO BALB/c background mouse models had to be postponed due to the COVID-19 pandemic, as there were Government regulations restricting access to laboratory and animal facilities during the height of the pandemic. I have therefore been unable to start on these experiments for my thesis.

In conclusion, T_3 can exert its actions in OFs through interaction with *THR/Thr*. Additionally, according to *in vitro* analysis, T_3 might indirectly involve in a GO development. Furthermore, the expression of TH transporter gene, *MCT8/Mct8*, in OFs is not mediated by T_3. However, hTSHR-A subunit plasmid immunization to BALB/c mice leads to *Mct8* upregulation. Therefore, TSAbs production might affect *Mct8* level in mOFs.

ABSTRACT

GD is a common autoimmune hyperthyroid disorder caused by the production of TSAbs. One of its extra-thyroidal clinical manifestations is GO, which is an autoimmune disorder in the orbital region of eyes characterized by eye proptosis, diplopia, and inflammation of the OFs. TSHR is present in the OFs and functionally activated by TSAbs. Therefore, it is now widely known that TSAbs link the autoimmune reaction in GD and GO. While GD patients with longer symptoms of hyperthyroidism carry higher risk to develop GO, the role of TH overproduction and accompanying hyperthyroidism in GO pathogenesis remains unknown. A reproducible preclinical mouse model of GD and GO was already established in the laboratory by plasmid electroporation encoding human TSHR A-subunit. The aim of this thesis was to elucidate the role of TH and its receptors in *in vitro* OF cultures established from patients and from the mouse model.

To address the aim, firstly the expression of *THR/Thr* and TH- responsive genes was examined in the two OF cultures. In addition, TH-responsive genes expression was assessed in OFs derived from KO mice lacking *Thra* or *Thrb* to determine if a specific *Thr* plays a more important role than the other on TH signalling in mOFs.

In the *in vitro* experiments with human and mouse OFs cultures, hyperthyroidism was artificially induced by overnight treatment of T_3. Both *THR* genes, namely *THRA* and *THRB* in human and *Thra* and *Thrb* in mouse OFs, were expressed. Importantly, *THRA*/*Thra* was predominantly expressed in both species of OFs. The examination of TH-responsive genes showed that T_3 treatment induces *KLF9* gene expression in GO OFs but only weakly induces *Klf9* gene in TSHR mOFs. Furthermore, *hr* gene expression was significantly induced in TSHR mOFs. The analysis on mOFs derived from KO mouse model lacking of *Thra* or *Thrb* revealed that *Klf9* and *hr* gene were not responsive to T_3. Finally, the expression of a TH transporter gene, *Mct8*, was significantly upregulated in mOFs derived from GD/GO mouse model compared to control β-Gal immunized mice.

In summary, the interaction between T_3 and its receptors in OFs was important to induce the expression of TH-responsive genes. However, T_3 might only indirectly involve in GO development. Furthermore, TSAbs production in BALB/c mice immunized with human TSHR-A subunit plasmid might have impact on TH transport to mOFs.

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APPENDIX

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APCs	Antigen presenting cells
β-Gal mOFs	Orbital fibroblasts derived from β-Gal-immunized mice
BAT	Brown adipose tissue
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAS	Clinical Activity Score
C/EBP	CCAAT/enhancer-binding protein
cDNA	Complementary deoxyribonucleic acid
Cq	Quantification cycle
CREB	cAMP-responsive element binding protein
CTLA4	Cytotoxic T-lymphocyte associated factor 4
DBD	DNA binding domain
DC	Dendritic cells
DEPC	Diethyl pyrocarbonate
DHT	5α-dihydrotestosterone
DIO1	deiodinase 1
DIO2	deiodinase 2
DIT	di-iodinated
DMEM	Dulbecco's modified eagle medium
dNTPs	Deoxyribose nucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial NO-synthase
ERK	Extracellular regulated kinase
EUGOGO	European Group of Graves' Orbitopathy
FCS	Fetal calf serum
FOXP3	Fork-head box P3
FSHR	Follicle-stimulating hormone receptor
FT_3	free 3,5,3'-triiodothyronine
GD	Graves' disease
GO	Graves' orbitopathy
GO OFs	orbital fibroblasts derived from GO patients
GPCRs	G protein coupled receptors
GPHRs	glycoprotein hormone receptors
GRKs	GPCR kinases
hADSC	human adipose derived stem cells
HIF-1A	hypoxia inducible factor-1a gene
HLA	Human leukocyte antigen
HLA-DR	MHC Class II surface receptor
hr	mouse hairless gene
HRP	horseradish peroxidase
HPT	hypothalamic-pituitary-thyroid
hTSHR	human thyroid stimulating hormone receptor
i.p.	intra-peritoneal

IFN	Interferon
IGF1R	insulin-like growth factor 1 receptor
IgG	immunoglobulin G
KLF9	human krüppel-like factor 9 gene
Klf9	mouse krüppel-like factor 9 gene
KO	knockout
LAT	Large neutral amino acid transporter
LATS	Long acting thyroid stimulator
LCHGR	lutropin choriogonadotropin receptor
LRRD	Leucine rich repeat domain
mAbs	monoclonal antibodies
MAPK	Mitogen activated protein kinase
mOFs	mouse orbital fibroblasts
MCT8	human monocarboxylate transporter 8 gene
mct8	mouse monocarboxylate transporter 8 gene
MHC	Major histocompatibility complex
MIT	mono-iodinated
MMI	2-mercapto-1-methylimidazole
M-MLV	Moloney Murine Leukaemia Virus?
MSC	mesenchymal stem cells
mTOR	Mechanistic target of rapamycin
NF-κB	Nuclear factor kappa-b
NIS	sodium/iodide symporter
NK	natural killer
Nrf2	nuclear factor erythroid 2-related factor
OATP	organic anion transporter polypeptide
OFs	orbital fibroblasts
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD-1	Programmed cell death 1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PGE_2	Prostaglandin E2
PKA	Protein kinase A
PKC	Protein kinase C
PLC-β	phospholipase C-β
PLZF	promyelocytic leukemia zinc finger
PPARγ	Peroxisome proliferator activated receptor γ
PPIA	human peptidylprolyl isomerase A gene
Ppia	mouse peptidylprolyl isomerase A gene
PTPN22	Protein tyrosine phosphatase 22
PTU	6-*n*-propyl-2 thiouracyl
pTreg	Peripheral regulatory T cells
PVN	Paraventricular nucleus
RT-qPCR	quantitative reverse transcription polymerase chain reaction
RNA	ribonucleic acid
ROS	Reactive oxygen species
RT	reverse transcriptase

RXR	retinoid x receptor
SD	serpentine domain
SEM	standard error of the mean
SLC10A1	sodium/taurocholate co-transporting polypeptide
SNPs	Single nucleotide polymorphisms
T_3	3,5,3'-triiodothyronine
T_4	3,5,3',5'-tetraiodothyroxine
TBAbs	TSHR blocking autoantibodies
TBII	Thyroid binding inhibiting immunoglobulin
TBG	Thyroxine-binding globulin
TCR	T cell receptor
Tg	Thyroglobulin
TPO	thyroid peroxidase
TT_4	total 3,5,3',5'-tetraiodothyroxine
TH	thyroid hormone
Th	Helper T cells
THR	human thyroid hormone receptor gene
THRA	human thyroid hormone receptor alpha gene
THRB	human thyroid hormone receptor beta gene
Thra	mouse thyroid hormone receptor alpha gene
Thrb	mouse thyroid hormone receptor beta gene
TMB	3,3',5,5'-Tetramethybenzidine
TNF	Tumor necrosis factor
TRE	thyroid hormone response element
Treg	Regulatory T cells
TNAbs	TSHR neutral autoantibodies
TSAbs	TSHR stimulating autoantibodies
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
TSHR mOFs	orbital fibroblasts derived from preclinical GD/GO mouse model
TSI	thyroid stimulating immunoglobulin
TRAbs	TSH receptor autoantibodies
TRH	Thyrotropin releasing hormone
TSI	Thyroid stimulating immunoglobulin
TTR	transthyretin
WT	wild type

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CURRICULUM VITAE

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