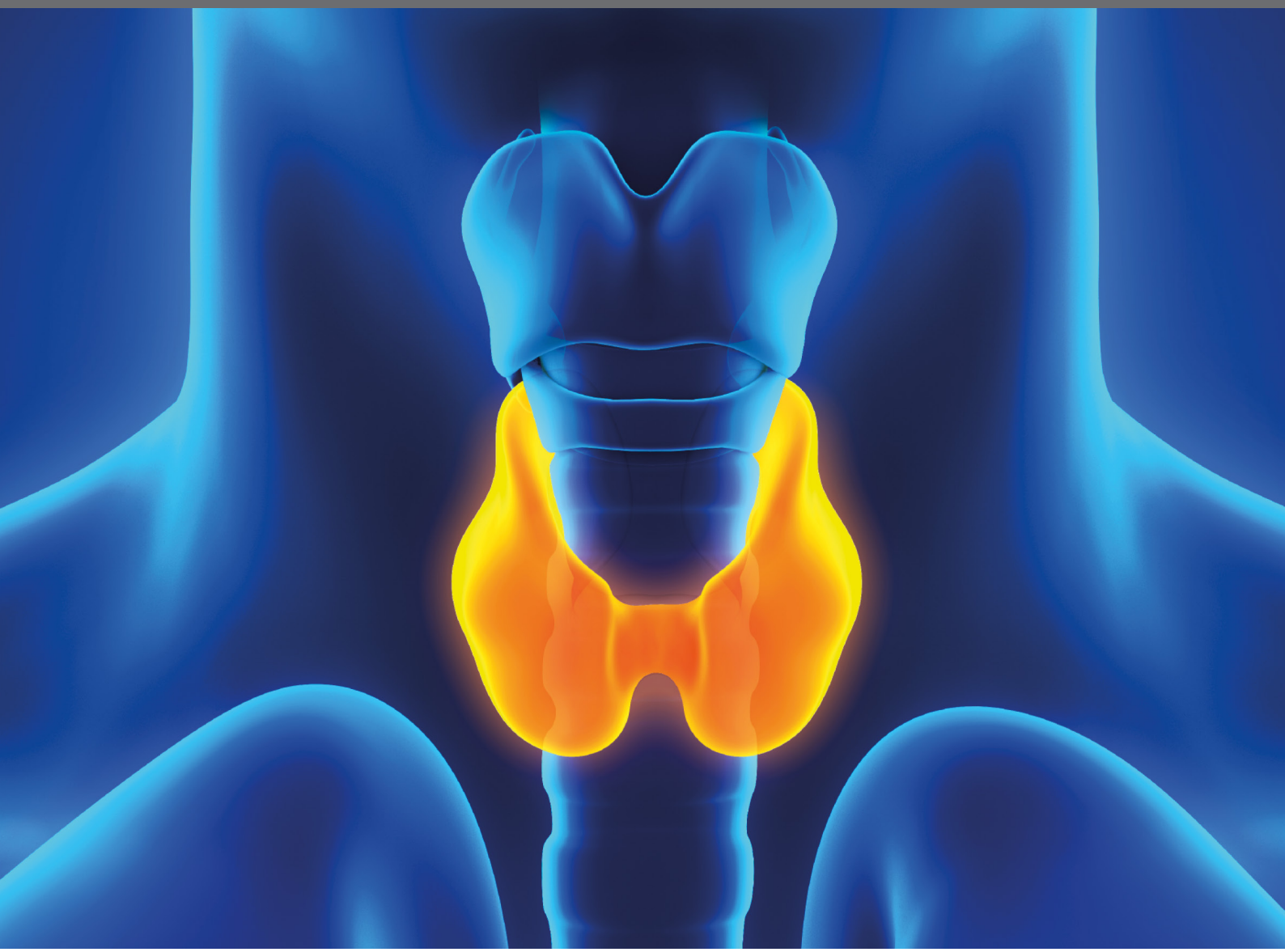
TSH RECEPTOR AND AUTOIMMUNITY

EDITED BY: Takashi Akamizu, Jae Hoon Chung, Cesidio Giuliani, Rauf Latif, Giorgio Napolitano and Susanne Neumann

PUBLISHED IN: Frontiers in Endocrinology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-816-5
DOI 10.3389/978-2-88945-816-5

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

TSH RECEPTOR AND AUTOIMMUNITY

Topic Editors:
Takashi Akamizu, Wakayama Medical University, Japan
Jae Hoon Chung, Sungkyunkwan University, South Korea
Cesidio Giuliani, University "G. D'Annunzio" Chieti-Pescara, Italy
Rauf Latif, Icahn School of Medicine at Mount Sinai, United States
Giorgio Napolitano, University "G. D'Annunzio" Chieti-Pescara, Italy
Susanne Neumann, National Institutes of Health, United Stated

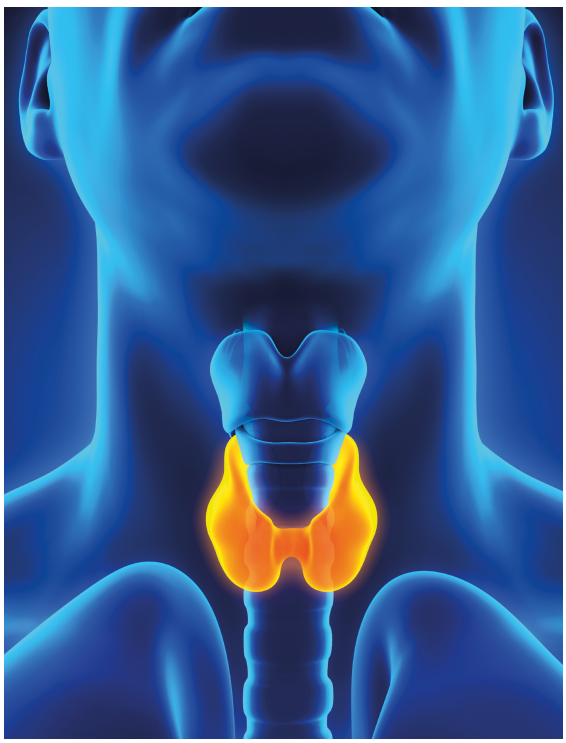


Image: Nerthuz/Shutterstock.com

The present Research Topic aims to provide updated reviews on TSH receptor structure and function, and its relationship with thyroid autoimmunity. Indeed, original reviews deal also with the pathogenesis of Graves' disease and its complications such as thyroid-associated opthalmopathy. Another issue that is addressed in this topic is the multifaceted nature of TSH receptor autoantibodies (TRAbs) and their role in the diagnosis and prognosis of Graves' disease, with the most recent data on the clinical applications of TRAbs assays. Furthermore, the functional role of the TSH receptor in extrathyroidal tissues is discussed.

Citation: Akamizu, T., Chung, J. H., Giuliani, C., Latif, R., Napolitano, G., Neumann, S., eds. (2019). TSH Receptor and Autoimmunity. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88945-816-5

Table of Contents

frontiers in Endocrinology

EDITORIAL
published: 30 January 2019
doi: 10.3389/fendo.2019.00019

Editorial: TSH Receptor and Autoimmunity

Terry F. Davies and Rauf Latif*

Thyroid Research Unit, The Mount Sinai Medical Center and the James J. Peters VA Medical Center, Icahn School of Medicine at Mount Sinai, New York, NY, United States

Keywords: TSHR (thyroid-stimulating hormone receptor), GPCR (G-protein-coupled receptors), autoantibodies, TSH (thyroid stimulating hormone), small molecule

Editorial on the Research Topic

TSH Receptor and Autoimmunity

INTRODUCTION

Over time it has become clear that the fascination with the TSH receptor (TSHR) is not only its complexity and its relationship to human disease but the fact that it keeps teaching us fundamental biology at all levels; cellular, molecular, and genetic. There are good examples of each of these facets in this cutting edge collection of papers. This contribution provides a brief and broad overview highlighting those areas of active progress by briefly eluding to some of the contributions in this collection.

The TSHR is a member of the class A family of G-protein coupled receptors (GPCR) with seven transmembrane helices traversing the plasma membrane and a large extracellular ectodomain. The ectodomain (ECD) is linked to a distal signal-specific domain—the hinge region—which is attached to a transmembrane domain (TMD) consisting of extracellular (ECL) and intracellular (ICL) loops (**Figure 1**). A partial TSHR ectodomain (residues 1–260) has been crystallized either bound to a stimulating TSHR antibody and/or a blocking TSHR antibody (1, 5) and recently in an unbound native state with stabilizing mutations. Like other GPCRs, the TSH receptor can also not exist in an ensemble of conformational states which can lead to its varied signaling potential. The review by Kleinau et al. in this collection takes a comprehensive look at the structure-function relationship of the TSHR via modeling and mutational approaches. It is now well-known that the full-length TSHR undergoes complex post translational processing (6, 7) inclusive of common protein modifications such as glycosylation and phosphorylation and even whole receptor modifications such as cleavage and multimerization (7, 8) thus resulting in a surprising variety of receptor configurations, many of which are expressed on the cell surface (9) and in some cases even shed from the cell surface (10). Although the shed receptor forms have not been conclusively demonstrated in the serum of patients with Graves' disease (GD), probably secondary to degradation, the evidence that these and other receptor structures are critical to the immunopathogenesis of GD has been well-covered in the review by Inaba et al.

Signal transduction at the TSHR is complex because of the promiscuous nature of the TSHR in engaging with different G proteins (11). In addition, the TSHR signals can be both G protein dependent and G protein independent. The TSHR has been shown to engage predominantly β-arrestin-2 for internalization (12) and arrestin-1, in human osteoblast cells, for differentiation, and MAP kinase signaling (13). In addition, it has long been known that the TSHR is involved with the IGF1/insulin receptor in thyroid cells and the "marriage" of these two receptors in fibroblasts has suggested their involvement in Graves' eye disease pathophysiology as well-reviewed by Smith et al.. The complex life cycle of GPCRs such as the TSHR (**Figure 2**)

OPEN ACCESS

Edited and reviewed by:
Derek LeRoith,
Icahn School of Medicine at Mount Sinai, United States

****Correspondence:***
Terry F. Davies
terry.davies@mssm.edu

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *24 December 2018*
Accepted: *14 January 2019*
Published: *30 January 2019*

Citation:
Davies TF and Latif R (2019) Editorial: TSH Receptor and Autoimmunity. Front. Endocrinol. 10:19. doi: 10.3389/fendo.2019.00019

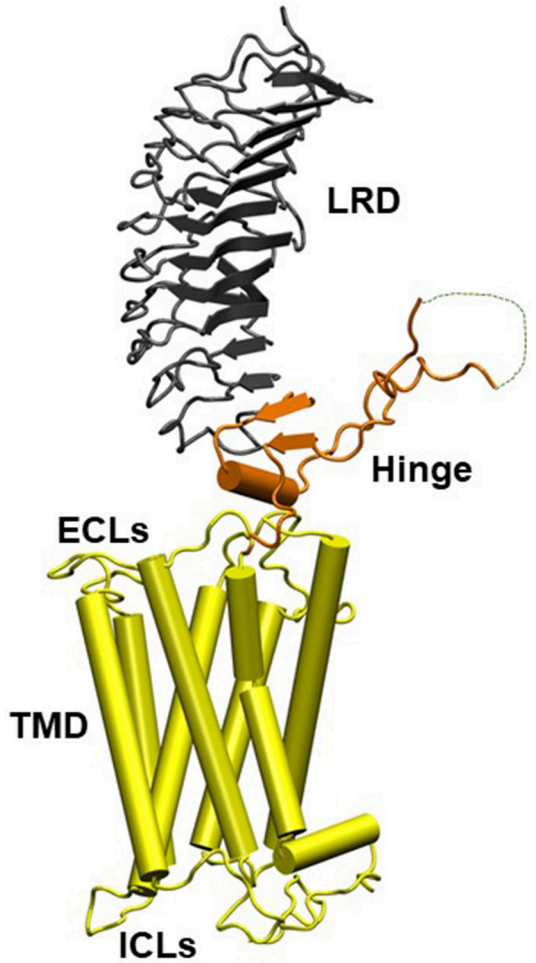


FIGURE 1 | Homology model of the entire TSH holoreceptor. This model highlights the tripartite structure of the TSHR. The ectodomain, shown in gray/black, is made up of 10 leucine-rich repeat domains (LRD) characterized as a "scythe-blade" shaped structure with loops and β pleated sheets obtained from the published crystal structure (1) (PDB:3G04). The region connecting the LRD and transmembrane domain (TMD), known as the "hinge" region, has recently been crystallized for the FSH receptor (2) (PDB:4AY9) and is shown as a looped structure (orange) with a helix conformation close to the carboxyl end of the LRD. The hinge in the TSHR has an additional sequence insert and is larger than in the FSH receptor. Therefore, amino acids 305-381 are missing in the illustrated model (3) and this insert is depicted as a closed dotted loop. The TMD (yellow), with its seven helices, is depicted as cylindrical structures connected to each other by the specific TSHR intra and extracellular loops. The TMD is the region that harbors the allosteric binding pockets for the SMLs. LRD, leucine-rich domain; TMD, transmembrane domain; ECL, extracellular loops; and ICL, intracellular loops [Figure adapted from (4)].

has also begun to be revealed showing that these types of GPCRs, after being sequestered via clathrin-coated pits or caveolin scaffolding proteins, are still able to signal after internalization. New evidence points out that these internalized receptors can lead to a "second wave" of signals from the TSHR (14). The result is that not only does the receptor come in multiple configurations but there are also multiple signal pathways that may or may not be initiated as the receptor conformation changes on ligand binding and this may continue after the receptors are internalized. The days of thinking simply of the TSH induced cyclic AMP response coming only from the surface receptors have long gone. Single-particle electron microscopy has confirmed the presence of intracellular megaplexes which consist of a GPCR bound to β-arrestin at its C terminus and a G protein complex at its core (15). The crystallization of a GPCR bound to G proteins has enhanced our understanding that ligands can stabilize different receptor conformations and that these ligand bound receptor complexes can stabilize different effector conformations leading to diversified signaling. However, such full-length receptor and G protein crystallized conformation(s) have not yet been achieved for the TSHR.

TSHR STIMULATORS

The TSHR can be activated by TSH itself, or by autoantibodies which can bind to the orthosteric site(s) on the large ectodomain. In fact, activation of the TSHR has been in clinical use for many years. Semi-purified bovine TSH was originally used for short-term thyroid testing of TSHR function but proved to have too many immune related side effects in clinical practice. The clinical use of TSH was not widely adopted until the introduction of recombinant human TSH in the 1990's. This is now used for detecting thyroglobulin release from metastatic thyroid cancer and for enhancing RAI uptake into thyroid glands (16–18). The discovery of stimulating TSHR antibodies by Adams and Purves (19) demonstrated the cause of Graves' disease and helped open up the entire field of autoimmune disease. Since the discovery of TSHR autoantibodies there has been the development of clinical assays to effectively detect these antibodies in Graves' patients with improving accuracy and sensitivity. The reviews by Giuliani et al. tracing the development of TSH bioassays and by Kahaly et al. on functionality and nomenclature are interesting and important in this regard. Although the current assays for detecting these antibodies are relatively robust the solid phase assays cannot detect bioactivity and the cell based bioassays are also not ideal where high concentrations of blocking antibodies may decrease the TSHR response to stimulating antibodies. Such problems arise due to the plethora of antibodies with variable bioactivities seen in GD indicative of a wide spectrum of variable activities as discussed further below.

In recent years it been shown by several investigators that selected small molecule ligands (SML) can easily permeate the plasma membrane and allosterically activate or inhibit TSHR signals. High throughput functional screening methods led to their identification and has opened up new therapeutic potentials (20–22). Furthermore, the concept that various effectors can stabilize the TSHR in a particular conformation has opened the possibility of biased TSHR signaling as achieved with other GPCR's (23, 24).

TSHR ANTAGONISTS

A major clinical need is for potent TSHR antagonists that can block the TSHR antibodies of hyperthyroid Graves'

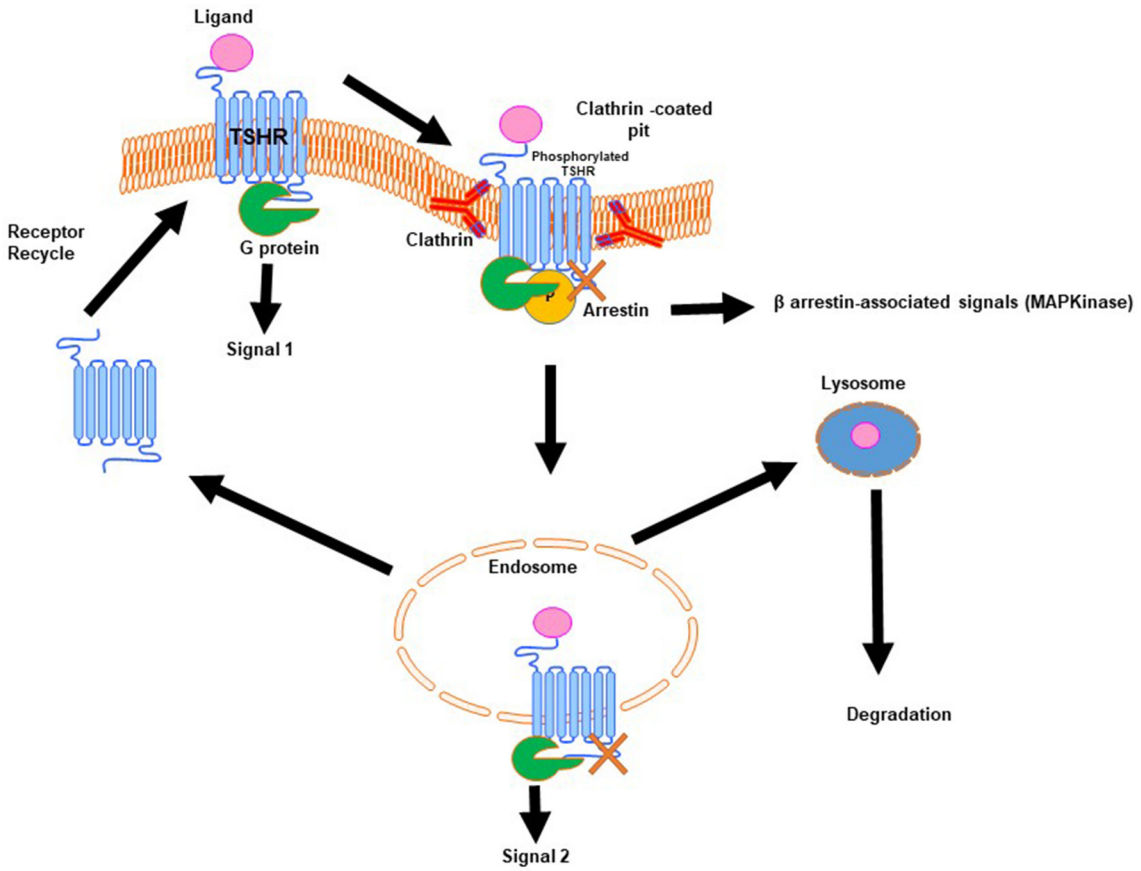


FIGURE 2 | Generic life-cycle of the TSH receptor. The TSHR residing on the plasma membrane of thyrocytes on binding with its cognate ligand, TSH, is activated and in turn undergoes conformational changes to recruit and activate a G protein complex leading to a predominate wave of Gs generated cAMP (**Signal 1**). The activated receptor, after signal 1, is phosphorylated and then moved to clathrin-coated pits where β-arrestin is bound to the activated receptor. At this stage it is believed that the receptor can signal via arrestin leading to β-arrestin-associated signals. Furthermore, this activated receptor in the invaginated pits is pinched-off to form the early and late endosomes. It is described that within the endosome the receptor with its associated ligand and second messengers is capable of giving out a second wave cAMP signal (**Signal 2**). Following this the receptor can be either degraded or it enters recycling vesicles and is recycled to the plasma membrane whereas the ligand is transported to the lysosome and is degraded. This is the life that the TSHR lives on the surface of thyrocytes or any other cell where it is expressed.

disease allowing us to dispense with the side effects of the common antithyroid drugs (methimazole and PTU) which deter many physicians from their long term use. A blocking human monoclonal TSHR antibody has been proposed as one method of achieving this aim (25) and results of a Phase II clinical trial are awaited. Although therapeutic antibodies have the theoretical advantage of specificity so do potential small molecule TSHR antagonists. Several groups, including our own laboratory as described by Latif et al. included in this collection (26, 27), have shown that allosteric inhibition of TSHR G protein signaling can silence the TSHR receptor. However, low potency and inadequate specificity of these SML antagonists indicate that more hurdles have to be crossed for the advancement of this approach. Peptide mimetics and aptamers to the TSHR that can either disrupt signaling via preventing G protein binding or by interfering with TSHR antigen processing are also under development and in early stage clinical trials and further data are awaited.

EXTRA-THYROIDAL TSHRs

At long last it is becoming widely known that the TSHR is expressed in more places than the thyroid gland and can even be found to be expressed in embryonic stem cells suggesting a role in development (11). The TSHR is expressed in fibroblasts, adipocytes, bone cells, and a variety of additional cell types (28, 29) and have, in particular, attracted a lot of attention in the retro-orbit (30–32) and bone (33, 34). This ubiquitous presence of the receptor clearly suggests that it has more functions than controlling thyroid hormone production. The role of TSHR activation and its signaling influence on adipocytes has been studied (35) and activation of the TSHR can modulate adipogenesis and fat cell phenotype further reinforced in the article by Draman et al.. The role of the TSHR in differentiation of preadipocytes into mature adipocytes from embryonic stem cells has also been shown (36) although the signals that influence this differentiation pathway are still unclear. The "Graves' Disease Triad" consists of hyperthyroidism with a dermopathy, referred

to as pre-tibial myxedema, and an orbitopathy often referred to as Graves' Eye Disease and involves fibroblasts and adipocytes at both extra-thyroidal sites. Retro-orbital expression of the TSHR, in combination with IGF-1 receptors (37), expressed on the fibroblasts and adipocytes behind the eye appear to be involved in the pathogenesis of Graves' orbitopathy GO- (see Smith et al.) and serum TSHR-Ab levels tend to correlate with eye disease (38–40). IGF-1 is well-known to enhance TSH action on thyroid cells and recent studies show that blockade of the IGF-1R appears to be a useful mode of therapy for GO (41, 42) presumably by reducing stimulating TSHR-Ab-induced adipocyte proliferation and cytokine release from retro-orbital fibroblasts. Such cytokines contribute to glycosaminoglycan generation and disrupt the osmotic pressure behind the eyes causing muscle fiber damage and swelling (42, 43). Similarly, our work on TSHR expression in osteoblasts and osteoclasts has identified TSH as a potential osteoprotective molecule (33). The identification of a TSH-β subunit splice variant secreted by bone marrow macrophages may be the effector of this protective effect as discussed in detail by Baliram et al. (44).

TSHR ANTIBODIES

One of the unique characteristics of Graves' disease, not found in normal individuals or in the rest of the animal kingdom, is the presence of TSHR antibodies (TSHR-Ab) which are easily detectable in the vast majority of patients as discussed earlier (45). In such patients, TSHR-reactive T cells and B cells survive central and peripheral deletion and under appropriate circumstances the B cells secrete TSHR antibodies and also induce T cells to secrete pro-inflammatory cytokines (46). Hence both B cells and T cells play a central role in mediating the chronic inflammatory changes of the autoimmune diseases seen in the thyroid gland, in the retro-orbit and in the skin (19), and may be resistant to T regulatory cell (Treg) control or allowed to be active secondary to inadequate Treg function (47). Although TSHR autoantibodies represent the hallmark of GD, finding the triggers that lead to this immunological derangement has been a challenge. Genome-wide association studies have established the association of the TSHR gene specifically with GD and understanding the functional mechanism by which such polymorphisms modify the physiological processes and trigger disease by interfering with central tolerance is outlined in the review by Stefan et al.. Whatever may be the major mechanisms for these triggers we now see three varieties of TSHR-Ab that can be found in patients with autoimmune thyroid disease and in TSHR immunized rodents; stimulating, blocking, and so called "neutral" antibodies; the latter often directed at the hinge region of the TSHR ectodomain and are far from being neutral in their biological activity. Stimulating antibodies induce cyclic AMP, thyroid cell proliferation and thyroid hormone synthesis, and secretion. They bind exclusively to conformational epitopes in the TSHR ectodomain leucine rich repeat region and compete with TSH for binding. TSHR blocking antibodies compete with TSH for binding and once bound they inhibit TSH action to a variable extent. However, the degree of blocking may be profound enough that they may induce hypothyroidism although some blocking TSHR antibodies may actually behave as weak TSHR agonists. In contrast, the neutral TSHR antibodies neither block TSH binding nor block TSH action but may be involved in aberrant signal initiation and thyroid cell apoptosis (48, 49). It is important to also remember that TSHR antibodies have an important role to play in pregnancy because these antibodies cross the placenta and influence both maternal and fetal thyroid function and their biochemical and immunological aspects are well-dealt with by Bucci et al..

APOPTOSIS IN GRAVES' DISEASE

It is now apparent that apoptosis plays an important role in the development and perpetuation of autoimmune thyroid disease. Areas of apoptosis are recognized in thyroid tissue from patients with Hashimoto's Thyroiditis and Graves' disease (50). Subsequent studies on apoptosis have provided insight into autoimmune target destruction, indicating the involvement of death receptors and cytokine-regulated apoptotic pathways in the pathogenesis, and perpetuation of thyroid autoimmunity. There is evidence that such thyrocyte apoptosis in Graves' disease may be antibody induced (51) or T cell mediated via defects in T regulatory cells which induce an abnormal production of cytokines (52) or changes in the expression of apoptotic molecules (Fas/FasL and caspase 8) on the surface of T lymphocytes and thyroid follicular cells (53, 54). In fact, all antibody binding to the thyroid cell induces thyroid cell stress, as first shown by our own laboratory, but we have shown that some neutral antibodies induce excessive ROS accumulation leading to thyroid cell apoptosis in the absence of G-protein signaling (49, 55, 56). This antibody induced apoptosis can facilitate the breakdown of self- tolerance mechanisms in individuals with the right major histocompatibility complex (MHC) class II background in myriad ways. It could be the release of excessive cytosolic DNA fragments that can act as adjuvants/immune modulators and induce aberrant MHC II expression in thyrocytes thus inducing the release of multiple inflammatory cytokines and chemokines as seen in various animal models and well-reviewed by Luo et al. in this collection.

THE MULTIPLICITY OF TSH RECEPTOR FORMS AND RESPONSES MAY EXPLAIN THE GRAVES' DISEASE PHENOTYPE

With the initial discovery of the classical G-protein–coupled-receptors (GPCR) the essential mechanisms appeared at first to be straightforward. The ectodomain was responsible for hormone specificity and the intracellular domain was responsible for the cyclic AMP signal. Each receptor had a specific ligand and an expected action. The receptor for TSH was very similar to that for FSH and LH/hCG and each activated PKA and the cyclic AMP pathway. Such simplicity, however, was short lived. Firstly, the TSHR was found to have two unique inserts into the ectodomain, including one which made it subject to complex post translational processing not seen with the LHR

and FSHR. Then the phenomenon of specificity cross-over reared its head. Suddenly the concept of high specificity of a hormone receptor was in doubt. For example, a number of ligands are able to bind to and activate the TSHR including hCG and LH. Stimulation of the TSHR by hCG is seen in gestational thyrotoxicosis (57) and in choriocarcinoma and a unique TSHR mutation even more highly hCG reactive has been described. With the burgeoning of our understanding into the structure of the TSHR by comparative modeling and partial crystal structures the entire field of TSHR signal transduction opened up. TSH/hCG and small molecule agonists could initiate different signals depending on the concentration of ligand available for receptor binding, the number of receptors activated, the forms of receptor (dimeric vs. monomeric) and also the orthosteric vs. the allosteric sites. Hence, we have the issue of multiple specificities and multiple signal responses indicating that an enormous number of variables are at play at just one GPCR. If we then consider Graves' disease and its multiple clinical forms which can vary from a highly localized thyroid disease to almost a systemic autoimmune diathesis much of this may be explicable by the variable forms of the receptor available for immune activation, the variable sites of TSHR expression and the multiplicity of signals that the TSHR can employ. In addition, the presence of differing proportions of high affinity TSHR-Abs with varied biological activity in patients with GD no doubt also contributes to the multiple clinical phenotypes; varying from hyperthyroidism to hypothyroidism and vice versa and with or without Graves' orbitopathy and pre-tibial myxedema.

CONCLUSION

The collection of papers that form part of this special issue shows the different facets of the TSHR thus allowing us to rightly say that many roads lead from and to this GPCR. For sure the TSHR, with its structural and signaling complexity, is going to hold our scientific imagination and enthusiasm for many more years to come.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by NIH grant DK069713, a VA Merit Award BX000800 (to TD) and the David Owen Segal Endowment.

REFERENCES

1. Sanders J, Chirgadze DY, Sanders P, Baker S, Sullivan A, Bhardwaja A, et al. Crystal structure of the TSH receptor in complex with a thyroid-stimulating autoantibody. *Thyroid* (2007) 17:395–410. doi: 10.1089/thy.2007.0034
2. Jiang X, Liu H, Chen X, Chen PH, Fischer D, Sriraman V, et al. Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. *Proc Natl Acad Sci USA.* (2012) 109:12491–6. doi: 10.1073/pnas.1206643109
3. Krause G, Kreuchwig A, Kleinau G. Extended and structurally supported insights into extracellular hormone binding, signal transduction and organization of the thyrotropin receptor. *PLoS ONE* (2012) 7:e52920. doi: 10.1371/journal.pone.0052920
4. Davies TF, Latif R. Targeting the thyroid-stimulating hormone receptor with small molecule ligands and antibodies. *Exp Opin Ther Targets* (2015) 19:835–47. doi: 10.1517/14728222.2015.1018181
5. Sanders P, Young S, Sanders J, Kabelis K, Baker S, Sullivan A, et al. Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. *J Mol Endocrinol.* (2011) 46:81–99. doi: 10.1530/JME-10-0127
6. Kursawe R, Paschke R. Modulation of TSHR signaling by posttranslational modifications. *Trends Endocrinol Metab.* (2007) 18:199–207. doi: 10.1016/j.tem.2007.05.002
7. Latif R, Morshed SA, Zaidi M, Davies TF. The thyroid-stimulating hormone receptor: impact of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on multimerization, cleavage, and signaling. *Endocrinol Metab Clin North Am.* (2009) 38:319–41, doi: 10.1016/j.ecl.2009.01.006
8. Urizar E, Montanelli L, Loy T, Bonomi M, Swillens S, Gales C, et al. Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. *EMBO J.* (2005) 24:1954–64. doi: 10.1038/sj.emboj.7600686
9. Chazenbalk GD, Tanaka K, McLachlan SM, Rapoport B. On the functional importance of thyrotropin receptor intramolecular cleavage. *Endocrinology* (1999) 140:4516–20. doi: 10.1210/endo.140.10.7031
10. Quellari M, Desroches A, Beau I, Beaudeux E, Misrahi M. Role of cleavage and shedding in human thyrotropin receptor function and trafficking. *Eur J Biochem.* (2003) 270:3486–97. doi: 10.1046/j.1432-1033.2003.03718.x
11. Davies TF, Ando T, Lin RY, Tomer Y, Latif R. Thyrotropin receptor-associated diseases: from adenomata to Graves disease. *J Clin Invest.* (2005) 115:1972–83. doi: 10.1172/JCI26031
12. Frenzel R, Voigt C, Paschke R. The human thyrotropin receptor is predominantly internalized by beta-arrestin 2. *Endocrinology* (2006) 147:3114–22. doi: 10.1210/en.2005-0687
13. Boutin A, Eliseeva E, Gershengorn MC, Neumann S. beta-Arrestin-1 mediates thyrotropin-enhanced osteoblast differentiation. *FASEB J.* (2014) 28:3446–55. doi: 10.1096/fj.14-251124
14. Godbole A, Lyga S, Lohse MJ, Calebiro D. Internalized TSH receptors en route to the TGN induce local Gs-protein signaling and gene transcription. *Nat Commun.* (2017) 8:443. doi: 10.1038/s41467-017-00357-2
15. Marshall FH. Visualizing GPCR 'Megaplexes' which enable sustained intracellular signaling. *Trends Biochem Sci.* (2016) 41:985–6. doi: 10.1016/j.tibs.2016.10.006
16. Huber GK, Fong P, Concepcion ES, Davies TF. Recombinant human thyroid-stimulating hormone: initial bioactivity assessment using human fetal thyroid cells. *J Clin Endocrinol Metab.* (1991) 72:1328–31. doi: 10.1210/jcem-72-6-1328
17. Ladenson PW, Braverman LE, Mazzaferri EL, Brucker-Davis F, Cooper DS, Garber JR, et al. Comparison of administration of recombinant human thyrotropin with withdrawal of thyroid hormone for radioactive iodine scanning in patients with thyroid carcinoma. *N Engl J Med.* (1997) 337:888–96. doi: 10.1056/NEJM199709253371304
18. Mazzaferri EL, Kloos RT. Is diagnostic iodine-131 scanning with recombinant human TSH useful in the follow-up of differentiated thyroid cancer after thyroid ablation? *J Clin Endocrinol Metab.* (2002) 87:1490–8. doi: 10.1210/jcem.87.4.8338
19. Adams DD, Purves HD. Abnormal responses in the assay of thyrotropin. *Proc Univ Otago Med School* (1956) 34:11–2.
20. Neumann S, Kleinau G, Costanzi S, Moore S, Jiang JK, Raaka BM, et al. A low-molecular-weight antagonist for the human thyrotropin receptor with

therapeutic potential for hyperthyroidism. *Endocrinology* (2008) 149:5945–50. doi: 10.1210/en.2008-0836
21. Neumann S, Gershengorn MC. Small molecule TSHR agonists and antagonists. *Ann Endocrinol.* (2011) 72:74–6. doi: 10.1016/j.ando.2011.03.002
22. Latif R, Ali MR, Ma R, David M, Morshed SA, Ohlmeyer M, et al. New small molecule agonists to the thyrotropin receptor. *Thyroid* (2015) 25:51–62. doi: 10.1089/thy.2014.0119
23. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* (1999) 286:2495–8. doi: 10.1126/science.286.5449.2495
24. Landomiel F, Gallay N, Jegot G, Tranchant T, Durand G, Bourquard T, et al. Biased signalling in follicle stimulating hormone action. *Mol Cell Endocrinol.* (2014) 382:452–9. doi: 10.1016/j.mce.2013.09.035
25. Furmaniak J, Sanders J, Nunez Miguel R, Rees Smith B. Mechanisms of action of TSHR autoantibodies. *Horm Metab Res.* (2015) 47:735–52. doi: 10.1055/s-0035-1559648
26. Neumann S, Nir EA, Eliseeva E, Huang W, Marugan J, Xiao J, et al. A selective TSH receptor antagonist inhibits stimulation of thyroid function in female mice. *Endocrinology* (2014) 155:310–4. doi: 10.1210/en.2013-1835
27. Latif R, Realubit RB, Karan C, Mezei M, Davies TF. TSH receptor signaling abrogation by a novel small molecule. *Acta Endocrinol.* (2016). 7:130. doi: 10.3389/fendo.2016.00130
28. Davies T, Marians R, Latif R. The TSH receptor reveals itself. *J Clin Invest.* (2002) 110:161–4. doi: 10.1172/JCI0216234
29. Marians RC, Ng L, Blair HC, Unger P, Graves PN, Davies TF. Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice. *Proc Natl Acad Sci USA.* (2002) 99:15776–81. doi: 10.1073/pnas.242322099
30. Bahn RS. Thyrotropin receptor expression in orbital adipose/connective tissues from patients with thyroid-associated ophthalmopathy. *Thyroid* (2002) 12:193–5. doi: 10.1089/105072502753600124
31. Bahn RS. TSH receptor expression in orbital tissue and its role in the pathogenesis of Graves' ophthalmopathy. *J Endocrinol Invest.* (2004) 27:216–20. doi: 10.1007/BF03345269
32. Gershengorn MC, Neumann S, Pope A, Geras-Raaka E, Raaka BM, Bahn RS. A drug-like antagonist inhibits TSH receptor-mediated stimulation of cAMP production in Graves' orbital fibroblasts. *Thyroid* (2012) 22:839–43. doi: 10.1089/thy.2011.0520
33. Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y, et al. TSH is a negative regulator of skeletal remodeling. *Cell* (2003) 115:151–62. doi: 10.1016/S0092-8674(03)00771-2
34. Baliram R, Sun L, Cao J, Li J, Latif R, Huber AK, et al. Hyperthyroid-associated osteoporosis is exacerbated by the loss of TSH signaling. *J Clin Invest.* (2012) 122:3737–41. doi: 10.1172/JCI63948
35. Elgadi A, Zemack H, Marcus C, Norgren S. Tissue-specific knockout of TSHr in white adipose tissue increases adipocyte size and decreases TSH-induced lipolysis. *Biochem Biophys Res Commun.* (2010) 393:526–30. doi: 10.1016/j.bbrc.2010.02.042
36. Lu M, Lin RY. TSH stimulates adipogenesis in mouse embryonic stem cells. *J Endocrinol.* (2008) 196:159–69. doi: 10.1677/JOE-07-0452
37. Iyer S, Bahn R. Immunopathogenesis of Graves' ophthalmopathy: the role of the TSH receptor. *Best Pract Res Clin Endocrinol Metab.* (2012) 26:281–9. doi: 10.1016/j.beem.2011.10.003
38. Khoo TK, Bahn RS. Pathogenesis of Graves' ophthalmopathy: the role of autoantibodies. *Thyroid* (2007) 17:1013–8. doi: 10.1089/thy.2007.0185
39. Kumar S, Nadeem S, Stan MN, Coenen M, Bahn RS. A stimulatory TSH receptor antibody enhances adipogenesis via phosphoinositide 3-kinase activation in orbital preadipocytes from patients with Graves' ophthalmopathy. *J Mol Endocrinol.* (2011) 46:155–63. doi: 10.1530/JME-11-0006
40. Kumar S, Schiefer R, Coenen MJ, Bahn RS. A stimulatory thyrotropin receptor antibody (M22) and thyrotropin increase interleukin-6 expression and secretion in Graves' orbital preadipocyte fibroblasts. *Thyroid* (2010) 20:59–65. doi: 10.1089/thy.2009.0278
41. Smith BR, Furmaniak J, Sanders J. TSH receptor blocking antibodies. *Thyroid* (2008) 18:1239. doi: 10.1089/thy.2008.0278
42. Bahn RS. Graves' ophthalmopathy. *N Engl J Med.* (2010) 362:726–38. doi: 10.1056/NEJMra0905750
43. Hansen C, Rouhi R, Forster G, Kahaly GJ. Increased sulfatation of orbital glycosaminoglycans in Graves' ophthalmopathy. *J Clin Endocrinol Metab.* (1999) 84:1409–13. doi: 10.1210/jc.84.4.1409
44. Baliram R, Chow A, Huber AK, Collier L, Ali MR, Morshed SA, et al. Thyroid and bone: macrophage-derived TSH-beta splice variant increases murine osteoblastogenesis. *Endocrinology* (2013) 154:4919–26. doi: 10.1210/en.2012-2234
45. Vlase H, Davies TF. Insights into the molecular mechanisms of the autoimmune thyroid diseases. In: Eisenbarth GS editor. *Endocrine and Organ Specific Autoimmunity*. R.G. Landes Co (1999). p. 98–132.
46. Bagriacik EU, Klein JR. The thyrotropin (thyroid-stimulating hormone) receptor is expressed on murine dendritic cells and on a subset of CD45RBhigh lymph node T cells: functional role for thyroid-stimulating hormone during immune activation. *J Immunol.* (2000) 164:6158–65. doi: 10.4049/jimmunol.164.12.6158
47. Dominguez-Villar M, Hafler DA. Regulatory T cells in autoimmune disease. *Nat Immunol.* (2018) 19:665–73. doi: 10.1038/s41590-018-0120-4
48. Ando T, Latif R, Davies TF. Antibody-induced modulation of TSH receptor post-translational processing. *J Endocrinol.* (2007) 195:179–86. doi: 10.1677/JOE-07-0058
49. Morshed SA, Ando T, Latif R, Davies TF. Neutral antibodies to the TSH receptor are present in Graves' disease and regulate selective signaling cascades. *Endocrinology* (2010) 151:5537–49. doi: 10.1210/en.2010-0424
50. Stassi G, De MR. Autoimmune thyroid disease: new models of cell death in autoimmunity. *Nat Rev Immunol.* (2002) 2:195–204. doi: 10.1038/nri750
51. Wang SH, Baker JR. The role of apoptosis in thyroid autoimmunity. *Thyroid* (2007) 17:975–9. doi: 10.1089/thy.2007.0208
52. Mao C, Wang S, Xiao Y, Xu J, Jiang Q, Jin M, et al. Impairment of regulatory capacity of $CD4^+CD25^+$ regulatory T cells mediated by dendritic cell polarization and hyperthyroidism in Graves' disease. *J Immunol.* (2011) 186:4734–43. doi: 10.4049/jimmunol.0904135
53. Bossowski A, Czarnocka B, Bardadin K, Stasiak-Barmuta A, Urban M, Dadan J, et al. Identification of apoptotic proteins in thyroid gland from patients with Graves' disease and Hashimoto's thyroiditis. *Autoimmunity* (2008) 41:163–73. doi: 10.1080/08916930701727749
54. Bossowski A, Czarnocka B, Bardadin K, Urban M, Niedziela M, Dadan J. Expression of Bcl-2 family proteins in thyrocytes from young patients with immune and nonimmune thyroid diseases. *Horm Res.* (2008) 70:155–64. doi: 10.1159/000145017
55. Morshed SA, Ma R, Latif R, Davies TF. How one TSH receptor antibody induces thyrocyte proliferation while another induces apoptosis. *J Autoimmun.* (2013) 47:17–24. doi: 10.1016/j.jaut.2013.07.009
56. Morshed SA, Latif R, Davies TF. Delineating the autoimmune mechanisms in Graves' disease. *Immunol Res.* (2012) 54:191–203. doi: 10.1007/s12026-012-8312-8
57. Glinoer D. Thyroid hyperfunction during pregnancy. *Thyroid* (1998) 8:859–64. doi: 10.1089/thy.1998.8.859

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared affiliation, though no other collaboration with the authors.

frontiers in Endocrinology

REVIEW
published: 24 April 2017
doi: 10.3389/fendo.2017.00086

Structural–Functional Features of the Thyrotropin Receptor: A Class A G-Protein-Coupled Receptor at Work

*Gunnar Kleinau[1,2], Catherine L. Worth[3], Annika Kreuchwig[3], Heike Biebermann[1], Patrick Marcinkowski[3], Patrick Scheerer[2] and Gerd Krause[3]**

[1] Institute of Experimental Pediatric Endocrinology, Charité-Universitätsmedizin, Berlin, Germany, [2] Group Protein X-Ray Crystallography and Signal Transduction, Institute of Medical Physics and Biophysics, Charité-Universitätsmedizin, Berlin, Germany, [3] Leibniz-Institut für Molekulare Pharmakologie (FMP), Berlin, Germany

The thyroid-stimulating hormone receptor (TSHR) is a member of the glycoprotein hormone receptors, a sub-group of class A G-protein-coupled receptors (GPCRs). TSHR and its endogenous ligand thyrotropin (TSH) are of essential importance for growth and function of the thyroid gland and proper function of the TSH/TSHR system is pivotal for production and release of thyroid hormones. This receptor is also important with respect to pathophysiology, such as autoimmune (including ophthalmopathy) or non-autoimmune thyroid dysfunctions and cancer development. Pharmacological interventions directly targeting the TSHR should provide benefits to disease treatment compared to currently available therapies of dysfunctions associated with the TSHR or the thyroid gland. Upon TSHR activation, the molecular events conveying conformational changes from the extra- to the intracellular side of the cell across the membrane comprise reception, conversion, and amplification of the signal. These steps are highly dependent on structural features of this receptor and its intermolecular interaction partners, e.g., TSH, antibodies, small molecules, G-proteins, or arrestin. For better understanding of signal transduction, pathogenic mechanisms such as autoantibody action and mutational modifications or for developing new pharmacological strategies, it is essential to combine available structural data with functional information to generate homology models of the entire receptor. Although so far these insights are fragmental, in the past few decades essential contributions have been made to investigate in-depth the involved determinants, such as by structure determination *via* X-ray crystallography. This review summarizes available knowledge (as of December 2016) concerning the TSHR protein structure, associated functional aspects, and based on these insights we suggest several receptor complex models. Moreover, distinct TSHR properties will be highlighted in comparison to other class A GPCRs to understand the molecular activation mechanisms of this receptor comprehensively. Finally, limitations of current knowledge and lack of information are discussed highlighting the need for intensified efforts toward TSHR structure elucidation.

Keywords: thyroid-stimulating hormone receptor structure, signal transduction, homology models, glycoprotein hormone receptors, arrestin interaction, G-protein interaction, structure–function relationships, oligomers

OPEN ACCESS

Edited by:
Rauf Latif,
Icahn School of Medicine at Mount Sinai, USA

Reviewed by:
Mihaly Mezei,
Icahn School of Medicine at Mount Sinai, USA
Efisio Puxeddu,
University of Perugia, Italy

****Correspondence:***
Gerd Krause
gkrause@fmp-berlin.de

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *02 February 2017*
Accepted: *03 April 2017*
Published: *24 April 2017*

Citation:
Kleinau G, Worth CL, Kreuchwig A, Biebermann H, Marcinkowski P, Scheerer P and Krause G (2017) Structural–Functional Features of the Thyrotropin Receptor: A Class A G-Protein-Coupled Receptor at Work. Front. Endocrinol. 8:86. doi: 10.3389/fendo.2017.00086

Abbreviations: GPHR, glycoprotein hormone receptor; LHCGR, lutropin/choriogonadotropin receptor; FSHR, follicle-stimulating hormone receptor; TSHR, thyroid-stimulating hormone receptor; TSH, thyroid-stimulating hormone; GPCR, G-protein-coupled receptor; TMH, transmembrane helix; ECL1/2/3, extracellular loops 1/2/3; ICLs 1/2/3, intracellular loops 1/2/3; SD, serpentine domain; CAM, constitutively activating mutation; WT, wild type; ECD, ectodomain; IP, inositol phosphate.

INTRODUCTION

The thyroid-stimulating hormone (TSH) or thyrotropin (1) receptor (TSHR) (2–6) is a member of the class A G-protein-coupled receptors (GPCRs) (7). Evolutionary close relatives are the two receptors for the gonadotrophic hormones: follitropin (FSH) (8) and lutropin (LH)/choriogonadotropin (CG) (9). The follicle-stimulating hormone receptor (FSHR) and the LHCGR together with the TSHR constitute the sub-family of glycoprotein hormone receptors (GPHRs) (10). The TSHR is essential for thyroid growth and function (11–13) and activates different G-protein subtypes (14–17) and signaling pathways (18–20), whereby Gs- and Gq-induced signaling are probably of highest importance (13, 21–24). TSH and its receptor are required for thyroid hormone synthesis and release in the thyroid gland (25). Dysfunctions of the TSHR are the underlying cause of various gain- or loss-of-function phenotypes associated with thyroid malfunction [reviewed in Ref. (26)]. It has been suggested that the TSHR is involved in the development and mechanisms of ophthalmopathy (16, 27–31).

For decades, the TSHR and associated molecular mechanisms, such as ligand binding (32, 33), cell-surface expression, or induced signaling cascades, were studied with the purpose to not only understand the different steps in signal transduction, their regulation, and specificity but also to receive insights into the related physiological aspects (13, 20, 34–38) or to develop tools for pharmacological treatment (39, 40). Consequently, a huge amount of specific data and information from genetic approaches (site-directed modifications), pathogenic conditions, protein structure studies, biochemical and biophysical analyses are available [see also the *information resource* of Sequence Structure Function Analysis for *GPHR* at http://www.ssfa-gphr.de (41–44) which contains >1,500 pathogenic and site-directed mutations; comparison of functional data enabled due to normalization as percentage of wild type (WT)].

This raises the following questions, what do we currently know about the complex scenario of signal transduction by the TSHR and what is currently far from our understanding? To answer these questions, here we summarize and discuss the current knowledge about the TSHR with a specific emphasis on structural aspects of receptor activation. This comprises the TSHR structure itself, complexes between this receptor and interacting proteins, and also the transition between different conformations related to different functional processes. For these purposes, the available—albeit fragmental—structural information for the TSHR and its interacting proteins will first be described followed by an assembling of this knowledge into homology models of the entire receptor highlighting the structural and functional specificities in relation to the signal transduction processes.

For understanding of "signal transduction" and related details described in the following sections, it is essential to keep in mind that the 3-dimensional TSHR structure is constituted by interplaying domains (**Figure 1**) located in different cellular environments. This fact is due to the principal molecular function of GPCRs as hubs to transduce signals. The "signal" is induced by ligand binding at the extracellular site and transmitted *via* structural rearrangements in the transmembrane-spanning receptor region [serpentine domain (SD) comprised transmembrane helices including their connecting loops] toward intracellular effectors. A receptor like the TSHR therefore not only receives a signal but it is also a trigger, catalyzer, and regulator for specific physical or biophysical information. Moreover, the communication inside the protein is regulated by several specific amino acids or groups of amino acids at diverse structural parts that are responsible, for instance, for intermolecular contacts (e.g., for

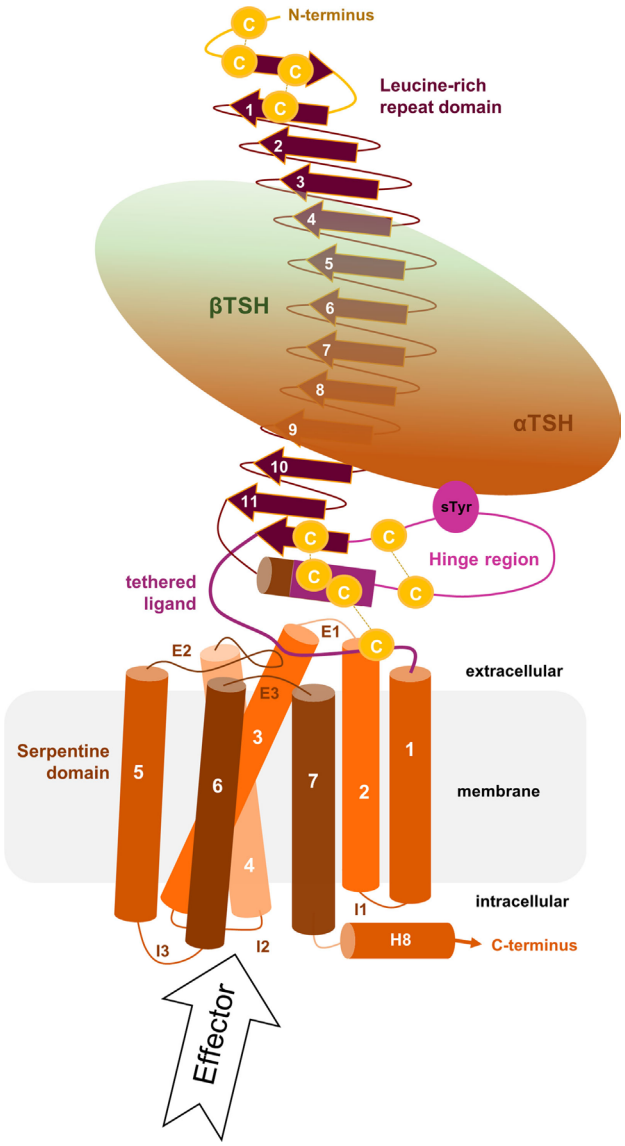


FIGURE 1 | Scheme of the putative overall thyroid-stimulating hormone receptor (TSHR) protein structure. This scheme shows the overall structure and domain assembly of the TSHR. Significant features are highlighted, e.g., the sulfated tyrosine in the hinge region that is involved in hormone binding. The leucine-rich repeat domain (LRRD) together with the hinge region constituting the extracellular receptor part. The seven transmembrane helices and their connecting loops arrange the serpentine domain, which spans the membrane from the extra- to the intracellular side. A tethered ligand located between the extracellular loops has been proven and is composed of amino acids from both C-terminal ends of the LRRD and the hinge region.

ligand binding) or intramolecular interactions (e.g., for maintenance of a specific conformation). In consequence, each part of the receptor has individual functional priorities that are interrelated with highly adapted structural features. The entire process of signal transduction is a sequence of concerted events that are disturbed under pathogenic conditions and must be circumvented by pharmacological interventions (45–52).

AVAILABLE STRUCTURAL INFORMATION

The Extracellular Leucine-Rich Repeat Domain (LRRD) and the Hinge Region

The extracellular LRRD and hinge region of the TSHR constitute the N-terminal extracellular receptor part (**Figure 1**), which is remarkably large (around 400 amino acids) compared to other class A GPCRs (10, 53). TSH and antibodies (activating, neutral, and blocking antibodies) interact with the receptor in this region [e.g., Ref. (54–58)]. The LRRD and the hinge region contain six asparagine-linked glycosylation sites (N-Xaa-S/T) that were already investigated intensively (59–62), and it was suggested that glycosylation of at least four sites appears necessary for expression of the functional TSHR (59).

The LRRD comprises repeats of specific amino acid sequences between 20 and 30 residues in length [for a detailed description of GPHR LRRD repeats, see Ref. (63)] known from available TSHR and FSHR crystal structures (56, 57, 64, 65) (**Figure 2**). The LRRD has a scythe blade-like shape with a slight twist from the N- toward the C-terminus. Hydrophobic amino acid side chains stabilize the inner core of the LRRD and aromatic interactions specifically are of high importance to maintain the backbone of the assembled repeats (**Figure 3**). Although the so far solved TSHR LRRD crystal structures showed a maximum of nine repeats (56, 57), based on homology modeling combined with mutagenesis studies (53), it was suggested that this domain is actually composed of 11 repeats (r1–r11 in **Figure 3**)—which was confirmed afterward by the recently solved FSHR LRRD structure (65). Interestingly, in contrast to other LRRDs with a similar fold (66–68), only the last C-terminal repeat of the GPHR LRRD is characterized by a short helix motif. Located in this helix are two cysteines at positions 283 and 284 that are known to interact with two cysteines at the C-terminal hinge region (65, 69). These disulfide bridges are important for adjusting both extracellular parts to each other and simultaneously anchoring the entire extracellular region close to the SD (**Figure 3B**). Moreover, gain-of-function mutations at position serine 281 leading to constitutive receptor activation were identified in patients (70, 71). This amino acid is also located in the helical part of the LRRD C-terminus and is crucial for activation (69, 70, 72, 73).

In the GPHR subfamily, the hinge region structurally links the LRRD with the SD (77). Unfortunately, little is known about the entire structure of the TSHR hinge region for several reasons. First of all, the TSHR hinge region is most likely not a self-folding domain (53). It might be that only parts of this region are specifically folded, or that interacting receptor fragments and/or the bound ligand are necessary to stabilize the hinge region in a specific conformation.

Related to this is the fact that the TSHR can be enzymatically cleaved at two sites in the hinge region (78, 79), which is also a prerequisite for shedding (78, 80–84) of the disulfide bridges located between the LRRD and the hinge region or inside the hinge region (**Figures 1** and **3B**). Shedding and cleavage in combination finally releases the so-called "receptor-subunit A" (constituted by the LRRD and parts of the hinge region) from the "receptor-subunit B" (C-terminal part of the N-terminus together with the SD) and cleavage plus shedding are unique to the TSHR in the group of GPHRs. This separation is likely related to the pathogenic occurrence of autoimmune antibodies against the TSHR (20, 34, 79, 85, 86). The cleaved peptide is termed "C-peptide" (approximately 50 amino acids in length), and it is still under debate how this process is related to physiological functions, signaling regulation, or pathogenic conditions (79, 84, 87–89). In any case, it is completely unknown how the C-peptide is folded or contributes to inter- and intramolecular interactions. This question remains important for understanding differences among the GPHRs.

From the crystal structure complex of FSHR ectodomain (ECD)/FSH only fragments of the hinge region are known, with a portion in the middle of the hinge region being unresolved (65). This missing part corresponds to TSHR residues 305–380. The entire TSHR hinge region is predicted to span positions 289–409 (53). However, the solved FSHR ECD crystal structure and derived models for the ECD TSHR (90, 91) highlight that the N- and C-terminus of the hinge region are essential for receptor functions like TSH binding and signal transduction. In detail, a third extracellular disulfide bridge between Cys301 and Cys390 [which is not conserved in GPHRs in general, reviewed in Ref. (9)] constrains the close interplay between the N- and C-terminus of the hinge region (**Figure 3B**). Cysteine 398 is located in a small beta-strand that is arranged parallel to the last beta-strand of the LRRD. This feature stabilizes the LRRD/hinge region complex, which may explain together with the two essential disulfide bridges Cys283/Cys398 and Cys284/Cys408 why this part was also solved in the FSHR crystal structure (65).

Moreover, the FSHR ECD crystal structure bound with FSH provided for the first time details of the second hormone-binding site of GPHRs around a conserved sulfated tyrosine (sTyr) (functionally corresponds to sTyr385 in TSHR). This tyrosine binds into a pocket between the hormone subunits and strongly contributes to hormone-binding properties (76), although small differences among the GPHRs were observed (92, 93). Generally, the hinge region of GPHRs is the least conserved receptor part (10, 63) and is therefore responsible for several differences concerning associated functions like hormone binding or induction of signaling pathways (94, 95).

The Membrane-Spanning SD

Currently, no structural information for the SD, comprising the seven membrane-spanning helices and respective connecting loops, has been experimentally determined yet for the TSHR or other GPHRs (**Figure 2**). This precludes detailed insights being made about amino acid interactions (at the atom level) and also the arrangement of the domains (SD, LRRD, and hinge region) or complexes to each other. However, it can be assumed that

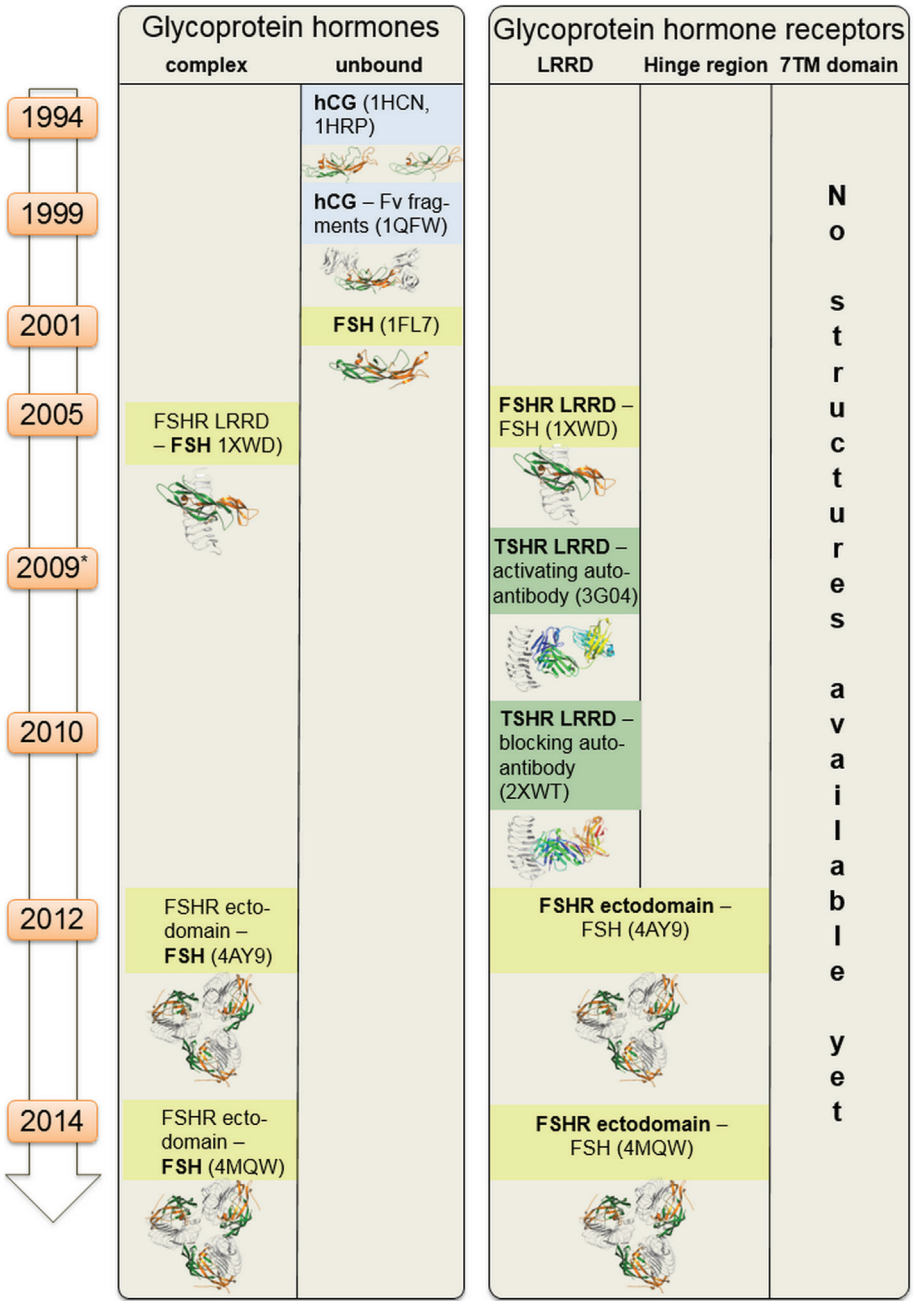


FIGURE 2 | Available structural information for glycoprotein hormone receptors (GPHRs) and GPHs. This scheme summarizes structural information that is available for the GPHRs and GPHs. Since 1994 starting with the first crystal structure of human choriogonadotropin, few further endogenous ligand structures (such as from follitropin or thyroid-stimulating hormone receptor autoantibodies—in complexes or unbound) were solved. Based on the high amino acid sequence similarity, each of these structures can also serve as structural templates for models of receptors and hormones where no structural information is available so far. Moreover, these structural data can be assembled into larger complexes (see **Figures 6**–**8**).

the TSHR has the same general assembly of the transmembrane helices as observed for all class A GPCRs because they share a common structural organization (96–99). Thus, experimentally determined structures of other GPCRs can be used as a proxy to generate TSHR models by using homology modeling techniques (100–103). This has been done several times in the past for different purposes [e.g., Ref. (45, 90, 104–107)]. These models were helpful for elucidating mechanisms of pathogenic mutations (26, 108, 109), allosteric small-molecule binding (45, 48, 49, 110), or G-protein and arrestin coupling (111) and guided more rational experimental approaches by suggesting potential interactions or mechanisms, in advance of already available knowledge. These experiments, in turn, were useful for refining or proving model-based predictions.

How can a TSHR model based on already solved crystal structures of other GPCRs be generated? Initial attempts at building TSHR models used those GPCR crystal structure templates available at the time: (1) inactive conformations—rhodopsin [PDB entry 1F88 (112)], beta-2-adrenergic receptor [ADRB2, PDB entry 2RH1 (113), PDB entry 2R4S (114)]; (2) active

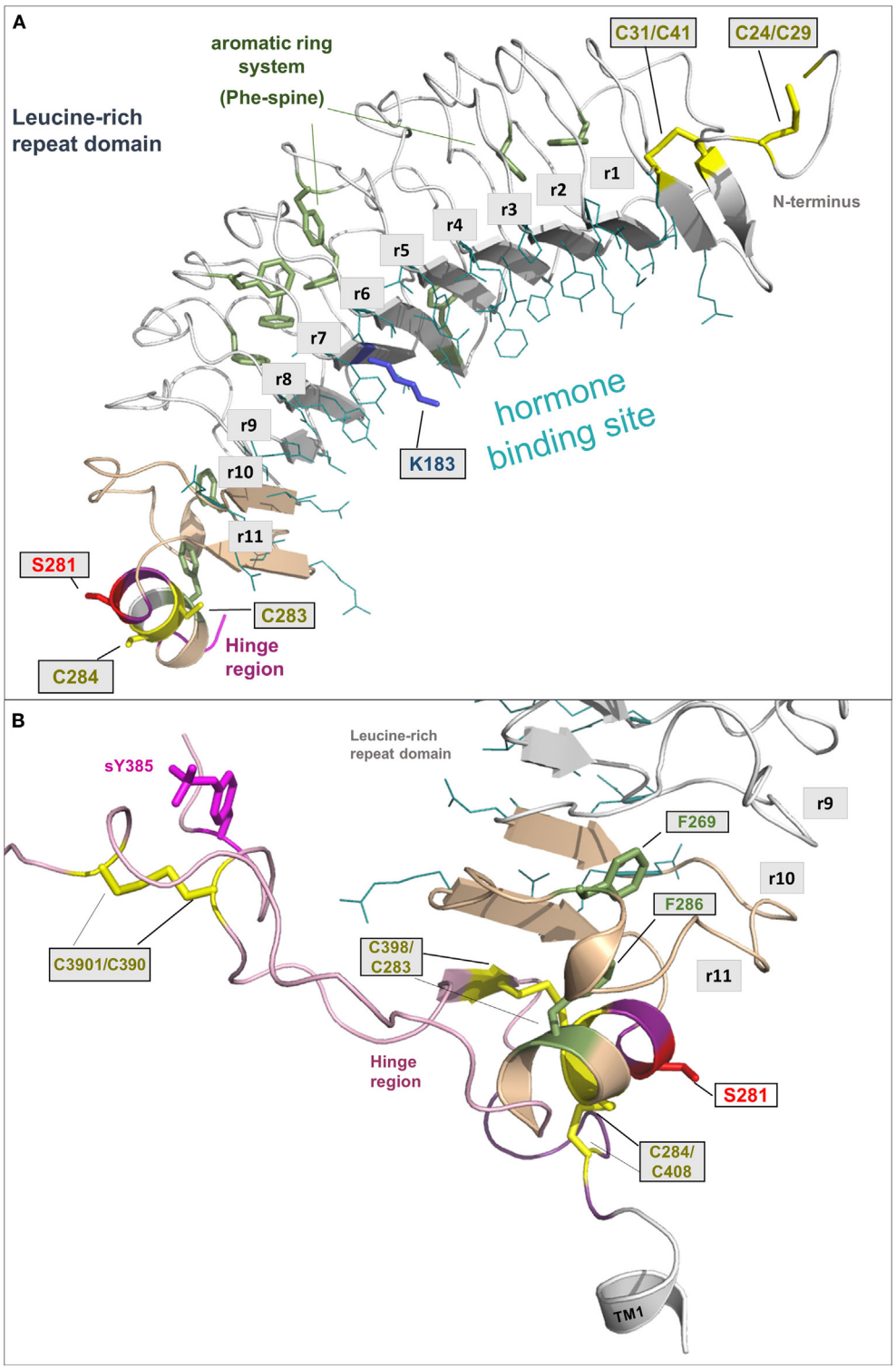


FIGURE 3 | Continued

FIGURE 3 | Continued

A full-length model of the thyroid-stimulating hormone receptor (TSHR) leucine-rich repeat domain and a fragmental model of the hinge region. **(A)** The LRRD of the TSHR is the main binding site for hormones and autoantibodies. They interact with amino acids in the concave site of this domain, which is arranged as a beta-sheet. Hydrophobic amino acid side chains are located mainly in the inner core of the domain, thus aromatic interactions are of high importance. Although the so far solved TSHR LRRD structures are constituted by a maximum of 9 repeats (56, 57), it was suggested (53) that this domain is actually constituted by 11 repeats (r1–r11)—as also presented here in this model (designed by a chimeric model-approach, LRRD model comprises amino acids 24–288). In contrast to other known LRRD structures with similarity to the glycoprotein hormone receptor (GPHR) LRRDs (66–68), the backbone on the convex side of this domain shows only one short helical structure namely in repeat 11. The cysteines at positions 283 and 284 are known to interact with two cysteines at the C-terminal hinge region **(B)**. Furthermore, mutations of serine 281 were identified as pathogenic (70, 71) and causing a gain of function by constitutive receptor activation. Of note, lysine 183 in repeat 7 (blue stick) was identified to be highly responsible for ligand specificity. The Lys183Arg substitution leads to a hypersensitivity for choriogonadotropin (74, 75). **(B)** This fragmental TSHR hinge region model (lilac-purple, amino acids 289–304 and 382–409) is adapted according to the solved follicle-stimulating hormone receptor (FSHR) ectodomain (ECD)/FSH complex structure (65) and contains several amino acids of high structural and functional importance. The cysteine 398 is located in a small beta-strand that is arranged parallel to the last beta-strand 11 of the LRRD. The two essential disulfide bridges Cys283/Cys398 and Cys284/Cys408 are shown. A third extracellular disulfide bridge between Cys301 and Cys390 stabilizes the interplay between the N- and C-terminus. Moreover, the recent FSHR ECD crystal structure bound with follitropin provided details for the first time on the second hormone-binding site of GPHRs around a conserved sulfated tyrosine (in TSHR sTyr385). This tyrosine binds into a pocket between the hormone subunits and contributes to ligand-binding properties (76).

conformations—opsin [PDB entry 3CAP (115)], opsin in complex with a C-terminal-binding peptide derived from the Gt-protein [PDB entry 3DQB (116)] or active metarhodopsin II (PDB entries 3PXO or 3PQR) (117), the beta-2 adrenergic receptor in complex with agonist and Gs-protein [PDB entry 3SN6 (118)], or the Adenosine-2A receptor in complex with an agonist and a mini-Gs protein [PDB entry 5G53 (119)]. The particular template selection was made based on the specific purpose of the models—like simulation of an inactive versus active conformation [e.g., Ref. (120)] and based on general or local sequence similarities. In the past decade, a large number of new crystal structures from diverse GPCRs were solved, including further aminergic receptors, chemokine, peptidic, or fatty acid receptors [reviewed in Ref. (102) and collected under http://gpcrdb.org/structure (121, 122)]. Consequently, this provokes the question as to what is currently the best structural template to model the SD or the entire structure of TSHR. Based on the overall sequence similarity, the closest single template for modeling the SD of TSHR is the beta-2 adrenergic receptor. However, primary sequence similarity to one single structural template may not be the best option. It is now common to build homology models using not only one template but using several template fragments in order to achieve maximum overlap of individual structural features, e.g., helical kinks or helical length dimensions (103, 123). Actually the TSHR has some of these specific structural properties related to amino acid fingerprints, which are not common in class A GPCRs. They are of high importance for an accurate model, and therefore they are also helpful to estimate the best modeling template. We will therefore extract and describe here a few significant examples important for defining structural properties of the TSHR, and we will also provide an inactive state model that is based on a "multi-fragment" approach (123).

One striking difference between the transmembrane helix (TMH) domain of most other class A GPCRs and the TSHR is that class A GPCRs typically contain a highly conserved proline in position 5×50 [modified Ballesteros and Weinstein nomenclature (124) considering structural alignments of bulges (125)] of TMH5, which is responsible for a bulged TMH5 conformation that causes a kink and twist toward the extracellular end of this helix. However, in the TSHR, there is an alanine (Ala593) in the corresponding position instead of a proline. Based on modeling approaches and mutant studies, in 2011 we suggested that an alanine at position 5×50 in TSHR causes a regular and stable alpha-helical conformation instead of a proline-supported bulge and kink in TMH5 (126). This structural prediction was later confirmed in crystal structures of receptors that do not have a proline at position 5×50 and which do indeed have a regular alpha-helical TMH5 such as the Sphingosine 1-phosphate receptor 1 [alanine in position 5×50; PDB entry 3V2W (127)], the P2Y12 receptor [asparagine in position 5×50; PDB entry 4NTJ (128)], and the lysophosphatidic acid receptor 1 [threonine in position 5×50, LPAR1, PDB entry 4Z34 (129)]. These structural implications for Ala593 in TMH5 of TSHR (126) were recently confirmed by others (104).

Moreover, a methionine (Met637) in TMH6 of TSHR is also a specific feature of this receptor because at the corresponding position (6×48) the majority of class A GPCRs have a highly conserved tryptophan. Replacement of Met637 by a tryptophan led to constitutive activation, indicating a different or altered side chain adjustment at this position in the TSHR (106). Homology models must be built by incorporation of these special functional–structural characteristics, ideally by using structures with the exact match in the respective property. The TMH5–TMH6 arrangement but also that between TMH3 and TMH5 are key features and should be significant for functionalities like the high basal signaling activity of the TSHR (130) or the huge amount of known constitutively activating TSHR mutations (26), whereby these structural features should predestine the TSHR for constitutive activation just by slight amino acid alterations.

To build the most accurate models with implementation of these specific features, a fragment-based modeling approach was developed, whereby templates are selected separately for each TMH and helix 8 using sequence fingerprint motifs and sequence similarity scores (103). The general aim was to select "best-choice" templates based on a logical decision tree or algorithm. This initial idea was transferred into a web server and database [GPCR-Sequence-Structure-Feature-Extractor (SSFE)[1]] to provide the tool to the larger community (123). This initial database contained pre-calculated models for more than 5,000 class A

[1]http://www.ssfa-7tmr.de/ssfe.

GPCRs (also including different species), but most importantly, this tool generates homology models and structural predictions for sequences of interest uploaded by the user. This method has recently been updated to include all 27 currently available inactive class A GPCR crystal structures for template selection and homology modeling.[2]

The inactive TMH model of TSHR generated during this recent update selected 6 of the 27 different template structures for model building (**Table 1**). Selecting transmembrane helices from different structural templates has the advantage that sequence differences causing slight backbone changes such as bulges or kinks are considered in more detail. Thus compared to using a single template, the multiple fragment approach can achieve an improved accuracy in the predicted models, which is essential for docking of small molecules or virtual screening. The reasons and fingerprint motifs for selecting particular TMH templates for the multiple fragment TSHR model are given in **Table 1**. For example, the conformation of TMH2 is based on TMH2 from ACM4 receptor (PDB entry 5DSG) since it contains (like TSHR) the fingerprint motif DXXXG at positions 2×50 to 2×54 and has the highest sequence similarity of similarly scoring templates. TMH3 of TSHR is based on TMH3 of AA2AR (PDB entry 4EIY) because of the matching fingerprint Gly–Cys at positions 3×24 and 3×25. TMH5 is based on TMH5 of LPAR1 (PDB entry 4Z34), since like TSHR, there is not only no proline in position 5×50 but also no Phe in position 5×47 and an Asn at that position instead. Three different templates OX1R (PDB entry 4ZJ8), OX2R (PDB entry 4S0V), and P2Y12 (PDB entry 4NTJ) score most highly for TMH6 and are suggested for modeling this helix. We selected the model using human orexin receptor type 1 (OX1R_HUMAN) for further analysis due to it having the highest number of motifs matched and having the best resolution for the X-ray structure. Thus, the resulting TSHR model contains distinct kinks in TMHs 2 and 6 and a straight TMH5 due to the matched fingerprint motifs in these helices (**Figure 4A**).

[2]http://www.ssfa-7tmr.de/ssfe2.

Figure 4B shows a comparison between this multiple fragment model with the best matching single template TSHR model based on the ADRB2 [PDB entry 2RH1 (114)]. The single template model differs not only by additional bulges in TMH2 and 5 and in the orientation of the highly conserved cysteine in TMH3 but also in orientations of the side chains Val421 (position 1×39) and Leu587 (position 5×44) (**Figure 4B**). Conservative mutations at these positions to isoleucine and valine, respectively, cause constitutive activation (104) and is thus incompatible with them being orientated toward the membrane as observed in the single template TSHR model (**Figure 4B**). However, the activating roles of these mutations are rationalized by the structural data when these side chains point toward neighboring helices (and thus potential interaction partners), as is observed in the multiple fragment TSHR model (colored in gray in **Figure 4B**). This clearly demonstrates the advantage of the multiple fragment approach in achieving an improved accuracy in the predicted SD models. Along these lines, recently 16 inactive crystal structures were used to generate multiple-template SD models of the TSHR utilizing another strategy (131). In their approach, Modeller (132) was used to build an averaged model of the TSHR SD by automatically combining all templates.

This also includes the intra- and extracellular loops. For adjusting the extracellular loops of TSHR models, different approaches have been used. SSFE integrated Superlooper2 (133), while others used Monte Carlo refinements (134) and Rosetta protocols (135) for TSHR loop modeling.

The SD model in an active state TSHR conformation can be built on the helix arrangement as observed in the crystal structures of opsin (116), metarhodopsin II (117), adenosine 2A receptor (119), or the beta-2 adrenergic receptor (118), where a huge outward tilt movement of ~8–14 Å of TMH6 were observed compared to the inactive state conformation [e.g., reviewed in Ref. (136, 137)]. The beta-2 adrenergic receptor crystal structure complexed with agonist and Gs-protein (PDB entry 3P0G) served as a template to build the TSHR active state SD model. However, additional TSHR-relevant fingerprints of TMH conformations (described above) were considered while modeling for TMH2 (kink but no bulge) and TMH5 (straight helix).

TABLE 1 | Template fragments from different G-protein-coupled receptor crystal structures used for building an inactive homology model of the serpentine domain of thyroid-stimulating hormone receptor.

Helix	Sequence similarity (%)	Suggested transmembrane helix (TMH) fragment template (UniProt entry name—PDB code)	Reasons for template selection (fingerprints)
TMH1	60	ACM2_HUMAN—3UON	Highest sequence similarity
TMH2	57	ACM4_HUMAN—5DSG	DXXXG at position 2×50 to 2×54, highest sequence similarity
TMH3	53	AA2AR_HUMAN—4EIY	GC at position 3×24 to 3×25
TMH4	50	OPSD_TODPA—2Z73	P at position 4×60, highest sequence similarity
TMH5	52	LPAR1_HUMAN—4Z34	No P at position 5×50, no F at position 5×47, N at position 5×47, highest sequence similarity
TMH6	47	OX1R_HUMAN—4ZJ8; OX2R_HUMAN—4S0V; P2Y12_HUMAN—4NTJ	No FXXCWXP motif at position 6×44 to 6×50, PXS at position 6×50 to 6×52, highest sequence similarity; no FXXCWXP motif at position 6×44 to 6×50, PXS at position 6×50 to 6×52, highest sequence similarity; no FXXCWXP motif at position 6×44 to 6×50, highest sequence similarity
TMH7	50	OPSD_TODPA—2Z73	Highest sequence similarity
H8	55	AA2AR_HUMAN—4EIY	Highest sequence similarity

Reasons and fingerprint sequence motifs for selecting a particular TMH template are given.

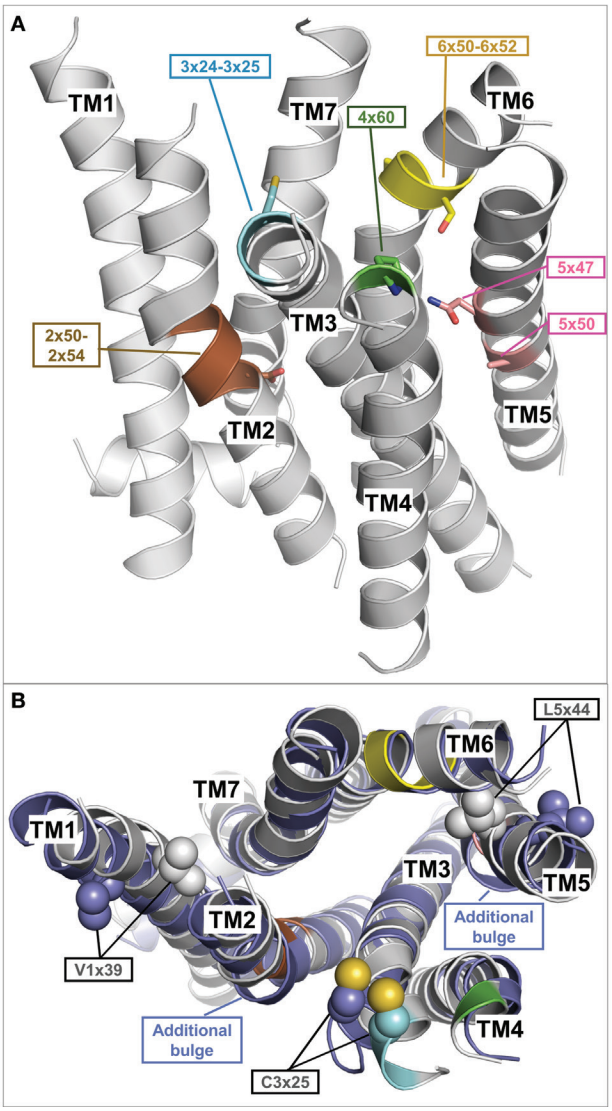


FIGURE 4 | Fragment-based thyroid-stimulating hormone receptor (TSHR) serpentine domain (SD) model with specific structural features. **(A)** This TSHR SD model was built from multiple transmembrane helix templates (see **Table 1**). The best matching fingerprint motifs between TSHR sequence and the selected template transmembrane helix (TMH) fragment are highlighted and indicate a central kink motif for TMH2 (brown: DXXXG at position 2×50 to 2×54), an extracellular kink for TMH3 (cyan: Gly–Cys at position 3×24 to 3×25), an extracellular proline for TMH4 (green: P at position 4×60), a regular central alpha helix for TMH5 (rose A5×50, N5×47), and a strong kink for TMH6 (yellow modified FXXCWP motif at position 6×44 to 6×50, PXS at position 6×50 to 6×52). The remaining TMH templates were selected based on having the highest sequence similarity. **(B)** Comparison of the multiple-template fragment-based model (gray) with the best matching single template TSHR SD model based on the beta-2 adrenergic receptor (PDB entry 2RH1) (blue), which differs in additional bulges in TMH2 and 5 but also in orientations of the side chains V421 (position 1×39) and L587 (position 5×44). Constitutively activating mutations of both residues (104) are rationalized by the fragment-based model when these side chains point toward neighboring helices (gray), but are incompatible with them being orientated toward the membrane as observed in the single template TSHR model (blue).

TSHR-Interacting Proteins—Hormones, Antibodies, G-Proteins, and Arrestin

The TSHR is a hub for signal transduction between different cellular regions and transduces information from signal inducers (extracellular) toward intracellular signaling molecules. Taking the high number of different GPCRs and ligands into consideration [more than 800 in humans (7, 138)], these ligand/GPCR(s)/effector systems are generally of high evolutionary success and importance (139). The physiological differentiation between particular GPCRs, their ligands, and resulting signaling in one cell or tissue are determined by time occurrence, cell-specific expression levels, ligand/receptor selectivity, and spatial separation, which also holds true for the TSHR under physiological conditions. In addition, for TSHR-interacting proteins like the Gs-protein (140–142) or TSH (143, 144) pathogenic mutants are known. These facts, as well as in context to its interacting proteins makes it very interesting to study and describe the TSHR or to search for further potential interaction partners that are unknown so far. But what is currently known about TSHR-interacting proteins in bound or unbound conformations?

In **Figure 5**, we provide an overview of known TSHR interaction partners and respective available structural information. In brief, TSHR can interact extracellularly with:

i. TSH and thyrostimulin, but no direct structural information is yet available, only structural homology models can be designed based on similarity to existing crystal structures of FSH [PDB entries 1FL7 (145)—unbound state, 1XWD (64) and 4AY9 (65)—bound state] or CG [all structures are in unbound state, PDB entries 1HCN (146), 1HRP (147), 1QFW (148)] (see **Figure 2**).
ii. Blocking [PDB entry 2XWT (57)] or activating antibodies [PDB entry 3G04 (56)], direct structural information is available in bound conformations, and also the unbound structure of an (inverse agonistic) antibody is available [PDB entry 4QT5 (149)].

In the transmembrane region TSHR can constitute:

iii. Homodimers (150, 151), which can be modeled by using several different GPCR dimer structures (see also Structural–Functional Aspects of TSHR Oligomerization), like from the μ-opioid-receptor [MOR (152)], κ-opioid receptor [KOR (153)], opsin (115), chemokine receptor CXCR4 (154), or the β-adrenergic receptor 1 [β-1AR (155)]. So far, it is unknown whether TSHR also constitutes functionally relevant heterodimers with other GPCRs, but it would be of enormous importance to clarify this question because heterodimerization could have dramatic consequences on TSHR functionalities as known from other GPCRs (156–160) and many different GPCRs are expressed in the same tissues as TSHR [e.g., searchable in Ref. (161)].

Intracellular interaction partners are:

iv. Arrestin, where bound complexes with opsin or rhodopsin are available [rhodopsin/arrestin PDB entries 4ZWJ (162),

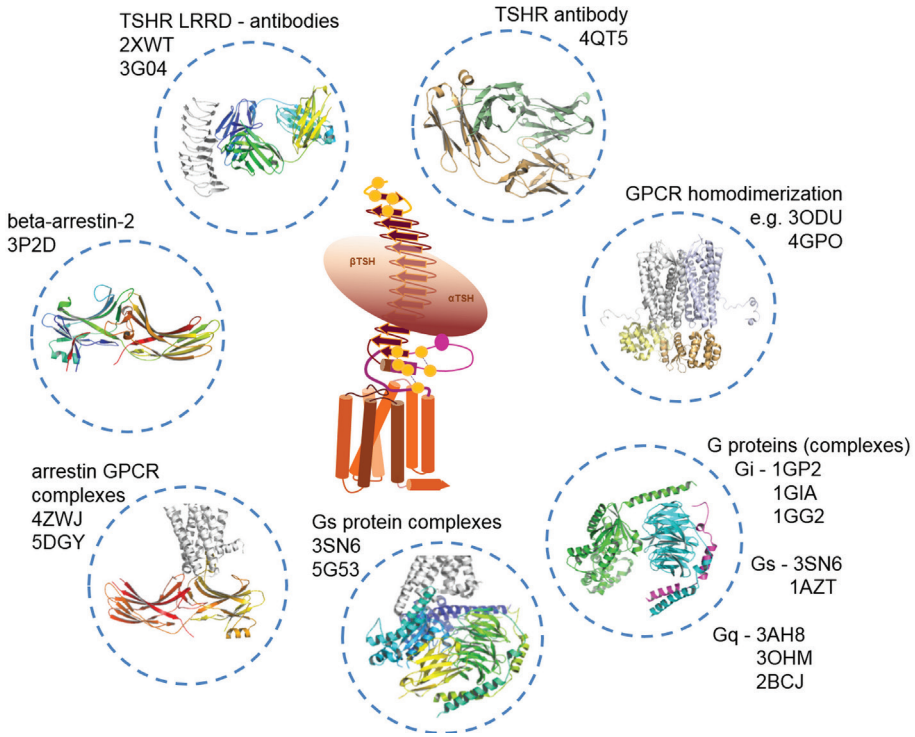


FIGURE 5 | Available structural information for thyroid-stimulating hormone receptor (TSHR) interaction partners. As shown in **Figure 2**, structural information on the TSHR is still limited. However, several interaction partners like autoantibodies (TSH is not solved so far), or Gi, Gs, and arrestin in bound and unbound conformations already have determined structures available. This knowledge can be used to construct larger model complexes as presented in **Figures 6**, **8** and **10**.

5DGY (163)], and opsin/arrestin fragment [PDB entry 4PXF (164)], but also unbound arrestin structures were already determined [e.g., inactive state—PDB entry 3P2D (165)], or pre-active states [PDB entries 4J2Q and 4JQI (166, 167)].

v. Numerous crystal structures of unbound (inactive) G-protein subtypes have been solved, like for Gi [PDB entries 1GIA (168), 1GG2 (169)], Gs [PDB entry 1AZT (170)], and Gq [PDB entries 3AH8 (171), 3OHM (172), 2BCJ (173)]. Based on the beta-2 adrenergic receptor/Gs complex, a bound Gs conformation is also available [PDB entry 3SN6 (118)].

Thus, based on the above as well as the information from **Figure 2**, it is clear that a specific set of structural information is already available for TSHR and interacting proteins, intracellular and extracellular. Consequently, the available data enables two objectives:

1. The assembling between TSHR and interacting proteins as models of complexes.
2. The estimation of structural transitions between the unbound and bound states for TSHR as well as for the interacting partners.

However, it must also be concluded that much structural information is still missing, such as from the TSHR-binding hormones [TSH, thyrostimulin (174–176)], or TSHR structures themselves, or with bound allosteric ligands or intracellularly complexed partners. Moreover, combined with the missing information of the entire TSHR SD region or the full-length receptor with spatially adjusted domains, the molecular interpretation of functional data from mutagenesis studies or pathogenic findings is an approximation rather than a definitive answer so far. However, in the following section, we describe examples of feasible complex models, which are based on above described structures or homology models.

Feasible TSHR and TSHR Complex Models

At the moment, the gap in structural information can only be resolved by building homology models based on the aforementioned crystal structures (**Figures 2**, **3A,B**, **5** and **9**). By building individual and complexed homology models, insight into the TSHR SD, the differences between active and inactive structures or between bound and unbound properties of the interacting proteins can be gained. The principal idea of homology modeling is to adapt the already determined homologous structures and respective amino acid sequences (e.g., described in Section "TSHR-Interacting Proteins—Hormones, Antibodies, G-Proteins, and Arrestin") toward the targets of interest—e.g., TSHR and TSH. This method is appropriate because the structural conservation and similarity of GPCRs is higher than their amino acid sequence similarity (100, 101, 103). We used the structural information documented above (i.–v.) to design

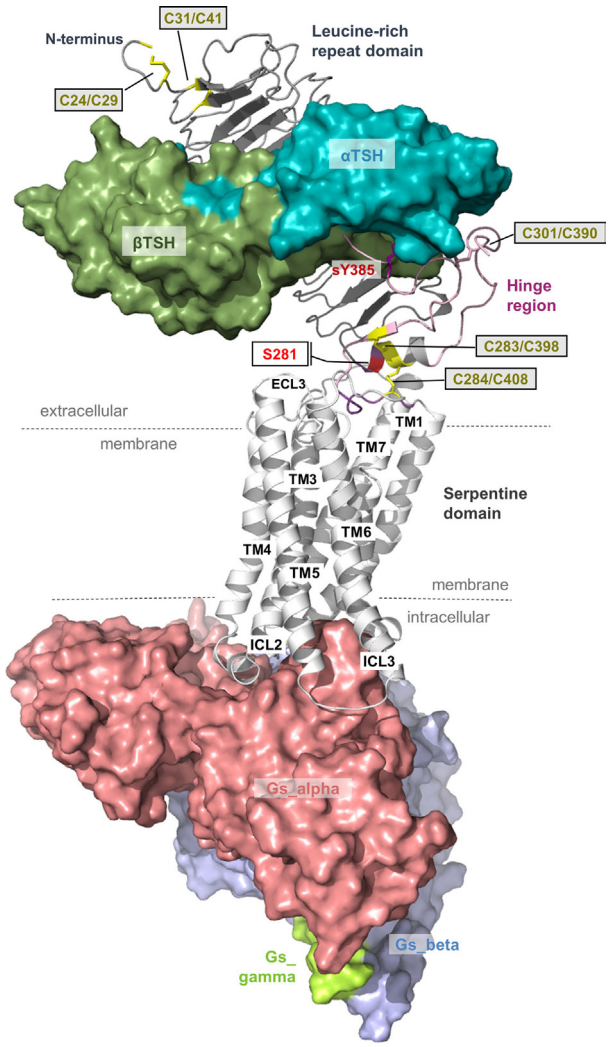


FIGURE 6 | A thyroid-stimulating hormone receptor (TSHR)/Gs complex model. The nearly completed complex model between TSHR–TSH and Gs in an active conformation can be assembled based on information summarized in **Figures 2** and **3**. TSH (or thyrostimulin) binds at two sites in the TSHR, called binding site I (LRRD) and binding site II (hinge region), of which several specific amino acids mediate the contact and specificity for the hormone. This model provides structural information according to the general TSHR scheme in **Figure 1**, including the detailed disulfide bridges at the extracellular part, localization of the hinge region, or justification of the Gs molecule at the active TSHR structure conformation [based on the beta-2 adrenergic receptor/Gs complex PDB entry 3SN6 (118)].

the following TSHR-related models in different activity-state conformations:

(1) The hormones TSH and thyrostimulin in bound and unbound conformations based on FSH (free and bound) or CG (unbound) (**Figures 2**, **6**–**8**).
(2) The full-length TSHR LRRD based on the LRRDs of the TSHR and of FSHR ECD/FSH complexes—as ligand bound conformations (**Figure 3**).
(3) The LRRD in combination with the hinge region based on the FSHR ECD/FSH complex—active state conformation (**Figures 3B** and **6**).

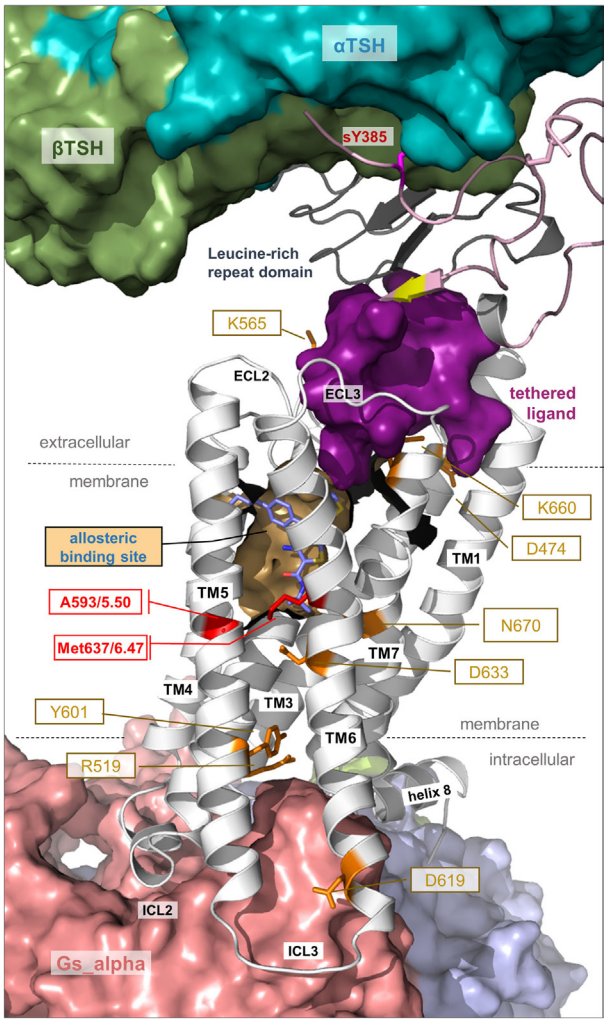


FIGURE 7 | Details of thyroid-stimulating hormone receptor (TSHR) structure and activation. This complex model visualizes important determinants and aspects of the TSHR activation mechanism. The hinge region links the LRRD with the serpentine domain and both parts harbor determinants for hormone binding. Ligand-binding triggers conformational changes at a convergent center between the LRRD and hinge region, thereby an inhibitory impact of the extracellular part on the receptor gets abrogated and an "intramolecular agonistic unit" or "tethered internal agonist" close to the transmembrane domain 1 becomes activated (violet surface). This extracellular signal induction is conveyed *via* structural rearrangements of the transmembrane-spanning helices toward the intracellular side. Several amino acids of high structural–functional relevance are involved in receptor activation (orange sticks) by maintaining specific activity-related conformations. They are localized at distinct spatial regions inside the TSHR, and they are interrelated with each other. The resulting active receptor conformation opens a spatial crevice for binding of intracellular interaction partners (**Figures 6** and **8**). Notably, the TSHR is characterized by specificities in the structural details such as a regular conformation of TMH5 compared to most other G-protein-coupled receptors (GPCRs), having an alanine instead of a proline at the 5×50 position, respectively. Moreover, the TSHR like all other glycoprotein hormone receptors (GPHRs) has a methionine at position 6×47 in TMH6, where usually a tryptophan is located in most class A GPCRs. In addition, it has been shown several times (48, 110, 177) that the known allosteric-binding sites for small drug-like molecules acting on GPHRs are located between the transmembrane helices close to the extracellular loops, which is shown here exemplarily by a partial surface-pocket representation and a bound synthetic antagonist.

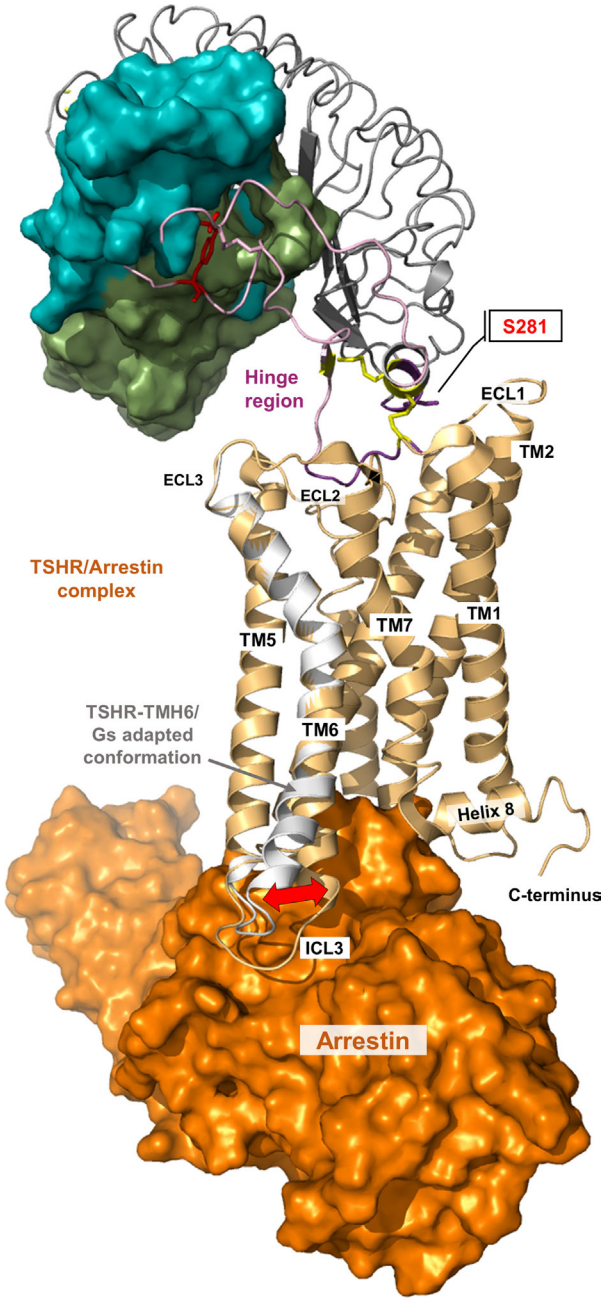


FIGURE 8 | A thyroid-stimulating hormone receptor (TSHR)/arrestin complex model. Binding and action of β-arrestin-1 and β-arrestin-2 on TSHR has already been reported (178–181). The putative structural conformation of TSHR adapted to this interacting protein is different to the TSHR/G-protein complex as shown in the presented superimposition of a TSHR/arrestin model (orange surface, complex is based on the crystallized rhodopsin/arrestin complexes PDB entries 4ZWJ, 5DGY) with the TMH6 conformation from the active TSHR/Gs complex (white backbone).

(4) The partial extracellular TSHR part bound with TSH or thyrostimulin based on the FSHR ECD/FSH complex (**Figure 6**).
(5) The TSHR extracellular part (LRRD and hinge region) bound with antibodies based on template chimeras between the solved LRRD/antibody complexes and the FSHR/ECD.
(6) TSHR SD in an inactive state (e.g., **Figure 4**) based on other GPCRs with determined structures.
(7) TSHR SD in active state conformations (e.g., **Figures 6**, **8** and **9**) like from ADRB2 or opsin.
(8) Inactive or active state conformations with bound allosteric ligands (**Figure 7**).
(9) TSHR SD or full-length TSHR as homomers (in inactive or active states) based on solved dimer structures of other GPCRs like opsin or MOR (**Figure 10**).
(10) TSHR in complex with arrestin (active state, **Figure 8**).
(11) TSHR in complex with G-protein (active state, **Figure 6**).
(12) TSHR homomers in complex with intracellular effectors [assembled active state complex models (**Figures 7** and **8**) in superimposition with dimeric GPCR crystal structures (**Figure 10**)].

These models provide insights into the:

- the putative structure and domain composition (**Figures 6–10**);
- hormone binding-related determinants (**Figures 3** and **6**);
- determinants of signal transduction at the extracellular region (**Figures 3** and **7**);
- constitution of the SD in different conformations (**Figures 6–9**);
- visualizing particular important amino acids for intramolecular signal transduction (**Figures 7** and **9**);
- TSHR-binding modes with G-protein or arrestin (**Figures 6** and **8**).

The models outlined above are advanced compared to the few experimentally determined TSHR structures yet they are only approximate models and not necessarily correct or precisely predictive. Functionally supportive data for assembling the SD and the extracellular region are rather rare (135, 189). More detailed methods for building these models are described in our own previous publications on the TSHR or other GPCRs [e.g., Ref. (53, 90, 91, 126, 189, 190)]. However, what can these models tell us or how can they help to visualize mechanisms of the TSHR? In the following sections, we will highlight several important insights related to regulation and action of the TSHR, which are strongly dependent on structural properties.

SIGNAL TRANSDUCTION BY STRUCTURAL REORGANIZATION: THE TSHR AT WORK

Induction of Signaling in the Extracellular Region

Induction of the endogenous signal transduction by the TSHR is triggered extracellularly by TSH (191) or thyrostimulin binding (174–176). The LRRD and the hinge region both harbor determinants for hormone binding [reviewed in Ref. (36, 63)]. Additionally, one specific residue of high importance for TSH binding is a sTyr sTyr385 (76, 92) located in the C-terminal end of the hinge region (**Figures 3** and **6**). Further amino acids in the hinge region are involved in ligand binding, mainly characterized by negatively charged side chains (53, 192–194). Generally, the hinge region has a drastic influence on hormone binding,

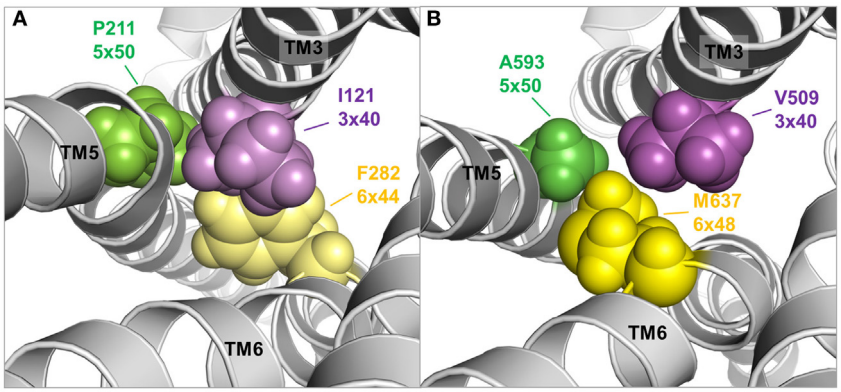


FIGURE 9 | TMH3–5–6 contact motif in the active state conformations of the beta-2 adrenergic receptor and an active state thyroid-stimulating hormone receptor (TSHR) model. A specific contact motif between residues in transmembrane helices 3, 5, and 6 is observed in the crystal structure of an active state conformation of the beta-2 adrenergic receptor (118) comprised Ile121 (3×40)—Pro211 (5×50)—Phe282 (6×48) [**(A)**, left panel]. Such hydrophobic contact can also be found in the TSHR model comprised Val509 (3×40)—A593 (5×50)—Met 637 (6×48) [**(B)**, right panel], although the amino acids differ. This contact motif is essential for triggering the active state in the TSHR.

structural constitution, and signal transduction, also in concert with the SD [e.g., Ref. (55, 195–201)].

From homology models of the TSHR (91) based on the crystal structure of the extracellular domain of FSHR (65), it was suggested that upon hormone or activating antibody binding a spatial displacement triggers conformational changes at a convergent center between the helical C-terminal end of the LRRD (pivotal helix) and the N- and C-terminus of the hinge region (**Figures 3** and **7**). The hinge region flexibility agrees with later suggestions that interactions between negatively charged residues in the hinge region and positively charged residues in the LRRD of TSHR are released upon hormone activation (202), or with suggested charged–charged interactions between the LRRD (Glu251) and hinge region (55). From the same models in 2012 (91), it also became clear that serine 281 is located within the short helix at the junction between the LRRD and hinge region (**Figure 6**). From naturally occurring mutations and mutagenesis studies, this residue is known to be functionally significant (constitutive receptor activation) (69, 70, 72, 73). This serine has also been suggested to interact with the extracellular loop 1 (73), which was recently supported by cross-linking studies (135).

Notably, the hinge region has an inhibitory function on receptor activity as revealed by previous mutational studies (201, 203–205). In addition, it was shown in 2002 that the extracellular N-terminal TSHR part switches from a tethered inverse agonist to an internal agonist (173), although the precise determinants of both (eventually separated) functional units are still not clarified in their entirety because of a lack of experimental structural data. However, in 2004, it was found that the internal agonist comprises specific amino acids (Asp403–Asn406) in the C-terminal hinge region (189) and further experiments refined these insights on the intramolecular agonist unit (196, 200). A recent study with a peptide including Asp403–Asn406 showed that it can act agonistically (90), providing evidence that the internal agonist (assumed for all three GPHR subtypes) is located extracellularly close to TMH1 (90, 189, 201). In conclusion, the TSHR is characterized by a tethered ligand, which is not common in class A GPCRs, but has been described as a mechanism in several particular cases (206). Moreover, the internal agonist is very likely embedded in-between the extracellular loops of the SD (90, 189, 201) and conveys the signal from the extracellular region toward the transmembrane domain (**Figure 7**). In this regard, it has been shown previously that the extracellular loops trigger the signal cooperatively (207).

Signal Transport across the Transmembrane Domain

Signal transduction by GPCRs is regulated by a specific rearrangement of particular helices to each other. But how does this process occur at the protein level and how is it regulated in the TSHR? Due to the lack of determined entire structures of the TSHR (and other GPHRs), the question arises how exactly does the extracellularly provided signal gives rise to helical movements. Generally, highly conserved amino acids in the class A GPCRs that are also found in the TSHR contribute to the maintenance of individual activity states and associated conformations by forming specific interactions. These interactions must be modified to facilitate helix movements and for new ones to occur after initial events to stabilize the active state conformation—in interplay with the ligand and the intracellular effector (208–211). It is known that the largest spatial movement related to GPCR activation affects TMH6 around a pivotal helix-kink at the highly conserved proline 6×50 (116, 118). This key event must also be assumed to occur in the TSHR, which is supported by the fact that a huge number of constitutively activating mutants, particularly on TMH6, are known for the TSHR (26, 43).

Moreover, both above described TSHR specificities—the regular alpha-helical conformation of TMH5 and the tightly packed methionine 637 in TMH6—have impact on the hydrophobic helix–helix interfaces between TMH3–TMH5–TMH6, which are important for the transition between the active and inactive

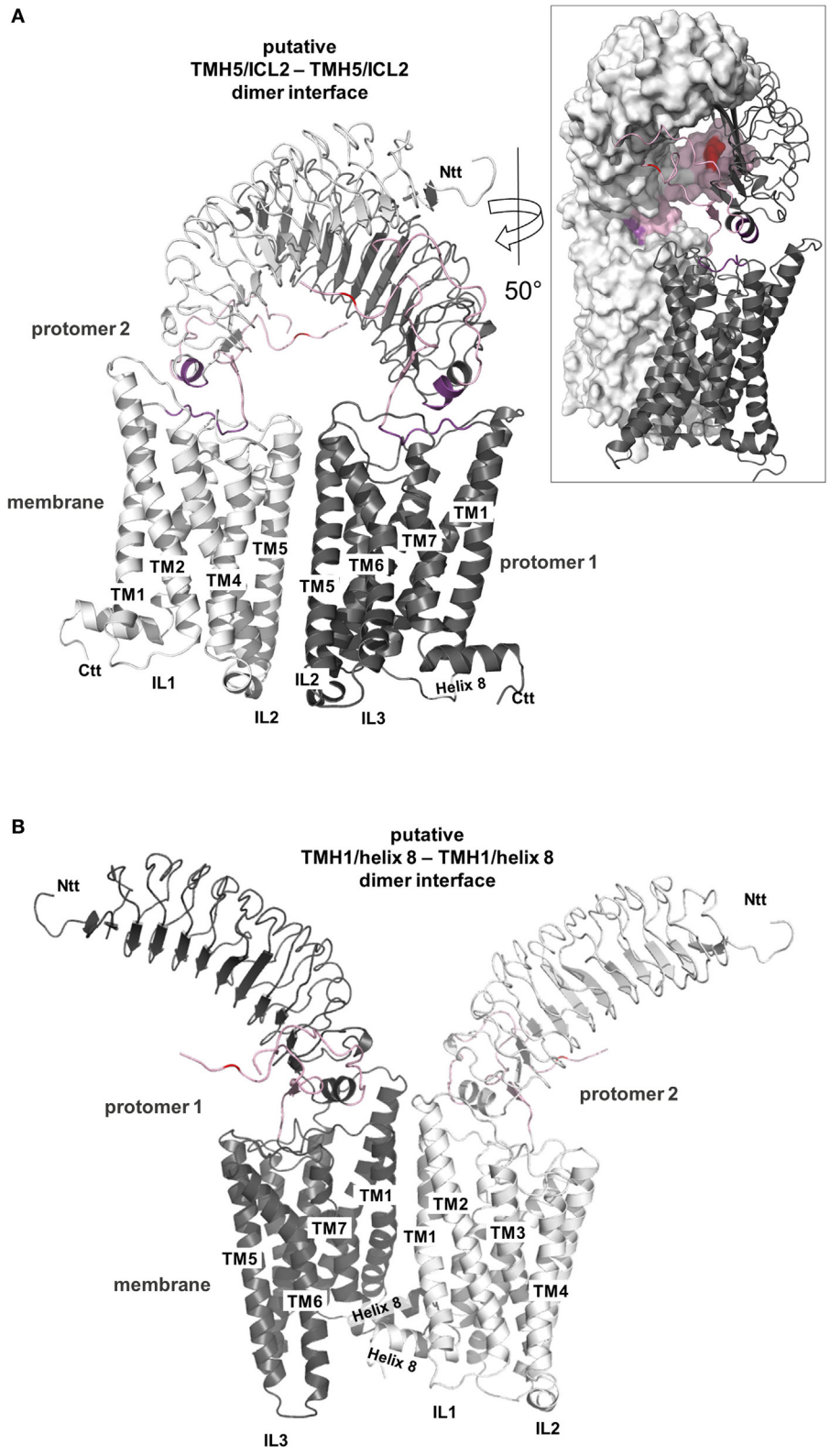


FIGURE 10 | Continued

FIGURE 10 | Continued

Putative thyroid-stimulating hormone receptor (TSHR) dimer formations. A definitive TSHR homodimer interface still awaits experimental evidence but based on the available data it can be summarized that the serpentine domain has the main impact on dimer formation with the extracellular part also contributing (182–184). Already crystallized G-protein-coupled receptor (GPCR) homodimer arrangements are available [reviewed in, e.g., Ref. (185, 186)] and they point to three different potential arrangements between the receptor protomers, at: (I) TMH1–helix 8/TMH1–helix 8, (II) TMH5–TMH6/TMH5–TMH6, and (III) TMH4–ICL2/TMH4–ICL2. These insights can be extrapolated to other GPCR oligomers assuming homology in sequence, structure, and mechanisms and using superimposition here we present two of these putative arrangements for a putative TSHR dimer constellation (150, 151, 187, 188) (entire homology model). In panel **(A)**, a putative TMH5/ICL2–TMH5/ICL2 interface is shown based on the solved dimeric chemokine receptor CXCR4 [PDB entry 3ODU (154)], and in panel **(B)**, a putative arrangement of the protomers with a TMH1/helix 8–TMH1/helix 8 interface is presented based on the opsin-dimer [PDB entry 3CAP (115)]. Both arrangements are feasible and also might occur simultaneously (e.g., in oligomers). In panel **(A)**, the extracellular parts of both protomers get sterically close (see insert with partial surface representation) and hormone binding would need a rearrangement of this extracellular constellation. In panel **(B)**, a symmetric TMH1–helix 8 interface hormone binding would not be influenced by the protomer arrangement.

state conformation. This is supported by previous studies where a hydrophobic interaction between TMH5 and TMH3 of the TSHR was analyzed by a complementary double mutant Val509Ala/Ala593Val (Val509, TMH3, 3×40; Ala593, TMH5, 5×50) (212). This double mutant led to a functional rescue of the respective single-mutant dysfunctions and provided evidence for a direct hydrophobic interaction of these TMH3 and TMH5 residues. This finding is strongly supported by crystal structures of other GPCRs in the inactive and active state conformation, where an inward movement of proline (in the corresponding position 5×50) toward TMH3 and 6 is observed for the active state such as for the beta-2 adrenergic receptor [ADRB2 (118)] or mu-opioid receptor [MOR (213)], thereby three hydrophobic residues of the ADRB2 located (i) on TMH5 (Pro211, 5×50), (ii) on TMH3 (Ile121, 3×40), and (iii) on TMH6 (Phe282, 6×44) interact tightly as a hydrophobic patch and contribute to the network of interactions that stabilize the active state conformation (**Figure 9A**). This spatial arrangement of the three hydrophobic residues was termed "PIF motif" or "contact motif" (210). Agonist binding induces these tightly packed hydrophobic interactions resulting in a rotation of TMH6, with a consequent outward tilt movement of the cytoplasmic helical end (**Figure 8**). Although the corresponding positions differ in sequence in the TSHR, a hydrophobic contact motif is also formed here by the aforementioned Ala593 (TMH5, 5×50) together with Val509 (TMH3, 3×40) and Met637 (TMH6, 6×48), which are subsequently also involved in the conformational active/inactive state transition (**Figure 9B**). This corresponds with constitutively activating mutations (CAMs) that were already identified at these TSHR positions [Ala593Asn (214), Val509Ala (212), and Met637Trp (106)].

But how are these modifications in the transmembrane region initiated or enabled? What we know is that the extracellular loops connect the helices (**Figure 1**) and it can be assumed that interactions occur between the TSHR hinge region and the extracellular loops (73, 201). They likely trigger the signal cooperatively toward the transmembrane region (207). In addition, specific loops or parts may also interact with the extracellular ends of certain helices as shown for the ECL2 and TMH6 in the TSHR (105). In conclusion, modifications of the loops can be transferred directly to interacting or connected helices, which are in line with reports in other GPCRs, where a salt bridge facilitates a link between the loops and receptor activation (215).

Second, signal transduction in the TSHR is not a single line of information flow but rather a multitude of synchronized sequences of events occurring. This assumption is made based on the fact that several previously reported inactivating or activating mutants at distinct amino acid positions are located at different receptor regions (**Figure 7**). Well investigated and significant examples are Lys660 in the TMH6/ECL3 transition (216), Lys565 in the ECL2 (105), Asp474 in TMH2 (217), or Glu409 in the transition between the hinge region and TMH1 (90) (**Figure 7**). Furthermore, Asp633 (TMH6) and Asn670 (TMH7) (107, 218, 219) are located in the central part of the domain core; and Tyr601 (220) or Asp619 (221, 222) is in the transmembrane region close to the intracellular site. In consequence and in contrast to the predominantly hydrophobic interfaces between TMH3–TMH5–TMH6, the helix–helix interfaces between TMH3, TMH2, TMH6, and TMH7 are characterized by the occurrence of essential hydrophilic contacts, e.g., at the highly conserved positions Asp2×50 or Asn7×50 (107, 219).

These hydrophilic contacts are complimented by conserved water molecules localized close to the mentioned conserved residues (103). Together, they constitute a network of intramolecular and water-mediated interactions (223) that are important for stabilizing GPCR structures by linking TMHs (224–226). Molecular dynamic simulations of class A GPCRs suggested an intrinsic water pathway, interrupted in the inactive state by hydrophobic layers of amino acid side chains, which change their conformation upon agonist binding leading to a continuous water channel. It is suggested that Tyr7×53 of the NPXXY motif is of importance in this context (227). Receptor activation probably leads to a rearrangement and an extension of the water network [for example, Ref. (90, 107)] from the ligand-binding site to the cytoplasmic surface (228, 229), at least for specific GPCRs. As well as water, allosteric sodium has also been observed in antagonist/inverse agonist bound class A GPCR structures in a highly specific arrangement between TMH2 and TMH7 (224). During activation, the sodium pocket collapses and the ion translocate toward the cytoplasm. However, it seems that not all GPCRs possess this pocket, such as visual opsins which instead have ordered water molecules between Asp2×50 and Tyr7×53 [PDB entry—4X1H (228)]. These observations underline the integral role of water molecules in GPCRs.

Apart from extracellular activation by its endogenous hormone ligands and autoantibodies, the TSHR signaling can be modulated by small-molecule ligands (SMLs) (52). Investigation of a potential allosteric-binding pocket for SMLs within the transmembrane domain (**Figure 7**) by modeling-driven mutagenesis led to the identification of distinct CAMs, including Val421Ile, Tyr466Ala, Thr501Ala, Leu587Val, Met637Cys, Met637Trp,

Ser641Ala, Tyr643Phe, Leu645Val, and Tyr667Ala (106), and silencing mutations such as Val424Ile, Leu467Val, Tyr582Ala, Tyr582Phe, Tyr643Ala, and Leu665Val (230). These positions not only indicate key amino acids covering the allosteric-binding pocket of the TSHR but also positions where the TSHR conformation can be changed to an active or inactive state. Mapping these residues onto a structural model of TSHR indicates locations where SML agonists or antagonists enhance or impair signaling activity (231). These signaling sensitive amino acids are also compiled in the web-based resource "SSFA-GPHR" (41–44).[3]

Intracellular Binding and Activation of Signaling Effectors

All amino acids of the intracellular TSHR loops were already investigated by site-directed mutagenesis studies (111, 221, 232–235). Moreover, pathogenic mutations at these receptor parts were also identified in patients (236–239). These mutants and site-directed studies revealed that the entire set of the three ICLs and helix 8 contribute to induction of intracellular signaling by the TSHR, although differences concerning the impact on specific signaling pathways has been observed. Diverse activation pathways in class A GPCRs converge near the G-protein-coupling region (240). In principle, GPCR-mediated G-protein activation is characterized by structural shifts inside and between the G-protein subunits to each other, followed by exchange of GDP for GTP in the alpha-subunit and (partial) separation of the Gα- from the Gβγ-subunits (241). This opens up interfaces to further contact partners (242). These events at the intracellular effector are induced by binding to the receptor in predisposition (without intracellular effector but with a bound agonist).

The intracellular effector can bind to the TSHR by fulfilling two criteria: (i) a spatial fit and (ii) an interaction surface that does not preclude binding, rather being supportive. So far, it is not known for GPCRs how exactly selectivity for a certain G-protein subtype is determined directly on the receptor. GPCRs with a preference for a particular G-protein subtype like Gs or Gq could not be allocated yet to a specific set of amino acids in the intracellular site. Additionally, receptor selectivity on the intracellular receptor site can be altered by making an amino acid substitution that repulses a specific effector (biased inactivation), and this is indeed the mechanism of several inactivating mutations in the intracellular TSHR loops, where, for instance, Gq activation is abolished but not activation of Gs [e.g., mutation Phe525Lys (243)]. This, in turn, would mean that selectivity is not associated with a complementary interaction pattern, it might be (theoretically) that selectivity in binding should be reached by a specific exclusion of effector subtypes due to small changes in the shape of the promiscuous receptor G proteins binding interface.

What is known concerning binding of intracellular effectors to the TSHR? As noted above, a huge amount of functional data from amino acid substitutions in relation to G-protein activation (not for arrestin binding) is already available and based on these data first molecular models of a putative TSHR/Gq-protein complex were previously generated (111). This can now be extended by incorporation of TSHR/Gs (**Figure 6**) and TSHR/arrestin (**Figure 8**) complex models based on recently determined structural complexes of other GPCRs [based on the beta-2 adrenergic receptor/Gs complex—PDB entry 3SN6 (118), or the rhodopsin/arrestin complex—PDB entry 4ZWJ (162)]. The intracellular loop 1 (ICL1) contributes to G-protein binding but the amino acids have a different impact (111). Of particular interest is Arg450 at the transition between ICL1 and TMH2, where several cases of naturally occurring inactivating mutations were reported (244–247). Amino acid Arg450 may directly interact with Gα as suggested by our homology model, e.g., with Gln390 in the C-terminal α5-helix of Gαs (111). However, the middle part of the ICL1 is exclusively oriented toward the beta-subunit of the G-proteins and mutations in this region only decrease inositol phosphate (IP) generation, not cAMP accumulation (Leu440Ala, Thr441Ala, and His443Ala). Of note, it was reported for the MOR that initial interactions between the G-protein and intracellular loop 1 and helix 8 may be involved in G-protein coupling specificity and that TMH5/6 contribute later in the process of complex formation (248). This finding would be in general agreement with our suggestion that ICL1 is also involved in G-protein coupling by the TSHR.

In addition, the intracellular loop 2 (ICL2) is significantly involved in G-protein activation in the TSHR (221, 243). Amino acids Met527, Arg528, and Asp530 are critical for both Gs and Gq activation, whereas alanine mutations of Ile523, Phe525, and Leu529 only impaired Gq-mediated signaling but not the Gs-mediated cAMP accumulation. Alanine mutations of Met527, Asp530, and Arg531 also caused impaired basal cAMP accumulation (120), which indicates involvement in Gs binding also in the basally active state conformation. Moreover, we suggest that the ICL2 conformation is helical (**Figure 6**) as supported by several crystal structures of diverse GPCRs, specifically in complexes (118, 162). In addition, the transitions between TMH5–ICL3–TMH6 were identified as being important for G-protein activation, whereby single substitutions of Tyr605, Val608, Lys618, Lys621, and Ile622 selectively decrease Gq activation (220, 221). By contrast, mutations at Asp617 and Asp619 cause constitutive receptor activation for the Gs-mediated pathway (218, 221, 239, 249).

Finally, these mutation-based studies at all three ICLs have shown that the binding modes between TSHR and Gs versus Gq do partially overlap, while completely inactivating mutations were only found for the receptor/Gq complex. The fact that Gq-mediated signaling, but not Gs-mediated cAMP accumulation, can be impaired by single side chain substitutions suggests that Gq binding is more fine-tuned than Gs binding. In strong relation to this might be the observed high basal activity for cAMP accumulation by TSHR, which is related to a permanent binding capacity and activation of Gs (130). The differences between Gs and Gq activation must be deciphered in more detail by determination of complex structures.

Moreover, so far, no experimental data from mutagenesis studies or structure determination are available concerning binding of arrestin to the activated TSHR, although arrestin binding is known to be of functional importance, e.g., for physically blocking further G-protein coupling and initiating the receptor

[3] http://www.ssfa-gphr.de.

shut-off (178–181). Activated GPCRs are phosphorylated by specific kinases on multiple sites at the C-terminus. In the inactive or basal state, arrestins are unable to bind activated TSHR, and interaction with several receptor-attached phosphates is critical for such an interaction. GPCR binding by arrestin is often discussed in terms of two events. Arrestin forms a low-affinity precomplex with the receptor, in which the phosphorylated receptor C-terminus replaces the C-tail of arrestin and thereby gains access to the high number of basic residues in the N-domain area (166, 167). C-tail displacement induces numerous conformational changes in key motifs and an overall domain rearrangement in arrestin that allow the second and tight-binding event of the activated receptor and the formation of a high-affinity complex. A key interaction of this high-affinity complex is the binding of the so-called finger loop region in arrestin to the intracellular-binding crevice of the activated receptor (162, 164), thereby the finger loop adopts a near helical structure and interacts with the highly conserved *E(D)RY* motif of the activated receptor. Remarkably, arrestin (namely, the near helical finger loop region) and G-protein (namely, the C-terminal alpha5 helix in the Galpha subunit) share a common binding crevice on the activated receptor (164). On the basis of the low-resolution crystal structure of peptide linker-fused rhodopsin–arrestin complex (162), a putative TSHR/arrestin complex model was created (**Figure 8**). The putative structural conformation particularly in TMH6 and ICL1-3 of TSHR adapted to this interacting arrestin model is slightly different to the TSHR/Gs-protein complex. However, until now, there are still many unanswered and unresolved questions due to the limited structural and biochemical knowledge of arrestin binding to GPHRs.

Structural–Functional Aspects of TSHR Oligomerization

Constitution of homo- and heteromers has been demonstrated for several members of different GPCR groups (250–253). Oligomerization is a biological tool for fine-tuning signaling and hence also physiological function (254–256), which is also relevant to endocrinology (257) and in pathological conditions (258–262). It is well documented that dimerization or oligomerization can have an impact on signaling properties as well as ligand binding (263, 264), signal transduction (265, 266), or cell-surface expression (267). Thus, oligomerization has been demonstrated to be a common and important feature of GPCRs including TSHR. What is known regarding TSHR oligomerization so far?

i. TSHR oligomerization (150, 151, 187, 188) occurs early in the endoplasmatic reticulum and is suggested to be crucial for proper receptor expression (268).
ii. TSHR probably forms higher order homomers rather than dimers (182) and the extracellular region participates in oligomerization, while the main protomer contact is most likely located at the transmembrane-spanning part (**Figure 10**) (183).
iii. A recent study revealed that two TSH molecules bound to a TSHR homodimer are required to activate not only Gs but also Gq (269).
iv. It has been debated as to whether TSH influences dimer formation (183, 270). On the one hand, it was proposed that oligomeric TSHR rapidly dissociates into active monomers upon TSH binding (271). On the other hand, dimerization was found not to be affected by ligand binding (182).
v. Functionally dominant-negative effects have been shown for partially inactivating TSHR mutations (272). TSHR di- or oligomerization presents a molecular explanation as to why these TSHR mutations exhibit a phenotypic effect even in the heterozygous state of an inactivating mutation (273).
vi. By contrast, CAMs do not influence dimeric TSHR arrangements (182, 274).

One of the basic questions concerns TSHR oligomer organization from the structural perspective. Interfaces (contact-regions) between GPCR protomers were found under experimental conditions for different GPCRs, for instance, at the region of ICL2–TMH4 (275–277), TMH4–TMH5 (278), or TMH5–TMH5 (279–281). Most importantly, several crystal structures of dimeric GPCR complexes were determined, e.g., the μ-opioid-receptor [MOR (152)], κ-opioid receptor [KOR (153)], opsin (115), chemokine receptor CXCR4 (154), and the β-adrenergic receptor 1 [β-1AR (155)]. Dimer interfaces are observed between TMH5–6, e.g., in the crystal structure of the CXCR4, or in the case of opsin, KOR, and β-1AR, the protomer interface is located between TMH1 and helix 8. Due to these repeated findings in the dimeric crystal structures, it can be postulated that class A GPCRs tend to have a preference to form protomer contacts at TMH1, helix 8, TMH5, and the ICL2–TMH4 transition.

Detailed characterization of TSHR oligomerization pointed to the SD as a main determinant for intermolecular receptor–receptor interplay and indicated that the extracellular receptor region might participate in this constellation (183, 184, 282). Recent studies suggested that the TMH1 is a main contact in the SD of the TSHR (283), which is in accordance with several of the crystallized GPCR interfaces reported above [e.g., the KOR dimer interface at TMH1–helix 8; PDB entry 4DJH (153)]. In line with this finding and with the published crystalized dimers, we provide molecular homology models of two putative TSHR dimer arrangements (**Figure 10**). In a putative symmetric TMH5–TMH5 interface, the TSHR would have additional side chain contacts at the extracellular side between TMH5 and TMH6 (**Figure 10A**). In a putative contact arrangement between TMH1–helix 8 (**Figure 10B**), TMH2 would contribute to the protomer contacts. A striking difference between both general orientations of the protomers is the relative orientation of the extracellular parts. Because it is so far unknown how the extracellular N-terminal LRRD and hinge region is arranged relative to the SD, the correct TSHR–TSHR constellation is unknown. According to our current homology models and arrangement of the ECD relative to the SD (**Figure 6**), a TMH5–TMH5 interface would result in sterical clashes between the extracellular parts and hormone binding would require initial structural modifications. In a TMH1–helix 8/TMH1–helix 8 protomer arrangement, the ECDs of both receptor molecules (models) would be freely accessible for the hormone molecules. In any case, it is reasonable to assume that both transmembrane interfaces occur simultaneously

in higher order complexes of the TSHR [as observed for the β-1AR (155)], which is probably functionally relevant for properties such as negative cooperativity in ligand binding caused by lateral intermolecular allosteric effects and/or negative intramolecular cooperative effects (183, 284).

Interestingly, the structure of the FSHR extracellular region with bound FSH was solved as a trimeric complex comprised three individual receptor/ligand units (49), while the previously solved FSHR/FSH complex with a shorter LRRD and without the hinge region (64) is a dimeric LRRD/hormone complex. Furthermore, in these two partial FSHR structures, interactions between the respective protomers are not similar, which might indicate flexibility in the arrangement or artificial constellations based on the crystallographic method. However, the trimeric-structure organization for GPHRs should be kept as one of various options for a multimeric receptor organization, since it also fits to several functional data (86, 285).

OPEN QUESTIONS AND FUTURE DIRECTIONS

In summary, well-defined structural rearrangements and interaction events between different proteins accompanies and characterizes the TSHR activation process. Any modification such as substitution of interacting amino acids may affect the resulting signaling, which is supported by a huge number of naturally occurring mutations in addition to designed inactivating or activating receptor mutants (41–44). Many insights concerning the TSHR structure in relation to detailed and general functions were already identified. This information is useful for deciphering the mechanisms of signaling or pathogenic conditions at the molecular level. However, we also draw attention to the lack of structural information, meaning that the main open questions concern the entire receptor structure—with and without the "C-peptide," with interaction partners (arrestin or G-proteins) or the exact oligomer constitution. For instance, the bound TSH structure in complex with TSHR would be hugely beneficial for many TSHR-related studies, including the improved directed development or refinement of medical therapeutics targeting the TSHR. Finally, the dynamic signaling process considering all known (and so far unknown) interaction partners resolved in time and cellular localization [also intracellularly (180, 181, 286–290)] would push the field enormously toward a comprehensive understanding of the TSHR, including suggested extra-thyroidal actions (29, 34, 291–296).

AUTHOR CONTRIBUTIONS

All authors have worked together on the manuscript in a back-and-forth procedure providing substantial contributions to the conception and interpretations. All authors have proofread the final version. In detail: GKleinau: conceptual contribution, major contribution to the content, generated homology models and their figures, and management of literature; CW: wrote modeling strategies especially for transmembrane domain modelling paragraphs, generated models and their corresponding figures, table, and checked English language; AK: wrote contributions about bioinformatics information resources and generated figures; HB: revised critical contributions about pathogenic and natural mutations of the TSHR; PM: wrote contributions about ligand binding and interaction; PS: wrote contributions concerning crystal structure interaction, especially concerning G-protein and arrestin interaction; GKrause: concept development, wrote and coordinated writing of the manuscript, and generated model figures.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (DFG), KR1273/4-2 to GKrause, BI 893/6-3 to HB, SFB740-B6 to PS, and DFG Cluster of Excellence "Unifying Concepts in Catalysis" (Research Field D3/E3-1) to PS.

REFERENCES

1. Mussett MV, Perry WL. The international standard for thyrotrophin. *Bull World Health Organ* (1955) 13:917–29.
2. Libert F, Lefort A, Gerard C, Parmentier M, Perret J, Ludgate M, et al. Cloning, sequencing and expression of the human thyrotropin (TSH) receptor: evidence for binding of autoantibodies. *Biochem Biophys Res Commun* (1989) 165:1250–5. doi:10.1016/0006-291X(89)92736-8
3. Nagayama Y, Kaufman KD, Seto P, Rapoport B. Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. *Biochem Biophys Res Commun* (1989) 165:1184–90. doi:10.1016/0006-291X(89)92727-7
4. Parmentier M, Libert F, Maenhaut C, Lefort A, Gerard C, Perret J, et al. Molecular cloning of the thyrotropin receptor. *Science* (1989) 246:1620–2. doi:10.1126/science.2556796
5. Frazier AL, Robbins LS, Stork PJ, Sprengel R, Segaloff DL, Cone RD. Isolation of TSH and LH/CG receptor cDNAs from human thyroid: regulation by tissue specific splicing. *Mol Endocrinol* (1990) 4:1264–76. doi:10.1210/mend-4-8-1264
6. Misrahi M, Loosfelt H, Atger M, Sar S, Guiochon-Mantel A, Milgrom E. Cloning, sequencing and expression of human TSH receptor. *Biochem Biophys Res Commun* (1990) 166:394–403. doi:10.1016/0006-291X(90)91958-U
7. Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* (2003) 63:1256–72. doi:10.1124/mol.63.6.1256
8. Simoni M, Gromoll J, Nieschlag E. The follicle-stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. *Endocr Rev* (1997) 18:739–73. doi:10.1210/edrv.18.6.0320
9. Troppmann B, Kleinau G, Krause G, Gromoll J. Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. *Hum Reprod Update* (2013) 19:583–602. doi:10.1093/humupd/dmt023
10. Vassart G, Pardo L, Costagliola S. A molecular dissection of the glycoprotein hormone receptors. *Trends Biochem Sci* (2004) 29:119–26. doi:10.1016/j.tibs.2004.01.006
11. Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, et al. Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. *Proc Natl Acad Sci U S A* (2002) 99:15462–7. doi:10.1073/pnas.242328999
12. Trunnell JB, Rawson RW. The effect of thyroid stimulating hormone on the function of human normal and malignant thyroid tissue. *J Clin Endocrinol Metab* (1948) 8:598.
13. Vassart G, Dumont JE. The thyrotropin receptor and the regulation of thyrocyte function and growth. *Endocr Rev* (1992) 13:596–611. doi:10.1210/er.13.3.596
14. Allgeier A, Offermanns S, Van Sande J, Spicher K, Schultz G, Dumont JE. The human thyrotropin receptor activates G-proteins Gs and Gq/11. *J Biol Chem* (1994) 269:13733–5.

15. Laugwitz KL, Allgeier A, Offermanns S, Spicher K, Van Sande J, Dumont JE, et al. The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. *Proc Natl Acad Sci U S A* (1996) 93:116–20. doi:10.1073/pnas.93.1.116
16. Wiersinga WM. Graves' orbitopathy: management of difficult cases. *Indian J Endocrinol Metab* (2012) 16:S150–2. doi:10.4103/2230-8210.104026
17. Van Sande J, Raspe E, Perret J, Lejeune C, Maenhaut C, Vassart G, et al. Thyrotropin activates both the cyclic AMP and the PIP2 cascades in CHO cells expressing the human cDNA of TSH receptor. *Mol Cell Endocrinol* (1990) 74:R1–6. doi:10.1016/0303-7207(90)90209-Q
18. Buch TR, Biebermann H, Kalwa H, Pinkenburg O, Hager D, Barth H, et al. G13-dependent activation of MAPK by thyrotropin. *J Biol Chem* (2008) 283:20330–41. doi:10.1074/jbc.M800211200
19. Krause K, Boisnard A, Ihling C, Ludgate M, Eszlinger M, Krohn K, et al. Comparative proteomic analysis to dissect differences in signal transduction in activating TSH receptor mutations in the thyroid. *Int J Biochem Cell Biol* (2012) 44:290–301. doi:10.1016/j.biocel.2011.10.024
20. Latif R, Morshed SA, Zaidi M, Davies TF. The thyroid-stimulating hormone receptor: impact of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on multimerization, cleavage, and signaling. *Endocrinol Metab Clin North Am* (2009) 38:319–41. doi:10.1016/j.ecl.2009.01.006
21. Kero J, Ahmed K, Wettschureck N, Tunaru S, Wintermantel T, Greiner E, et al. Thyrocyte-specific Gq/G11 deficiency impairs thyroid function and prevents goiter development. *J Clin Invest* (2007) 117:2399–407. doi:10.1172/JCI30380
22. Ledent C, Parmentier M, Maenhaut C, Taton M, Pirson I, Lamy F, et al. The TSH cyclic AMP cascade in the control of thyroid cell proliferation: the story of a concept. *Thyroidology* (1991) 3:97–101.
23. Verrier B, Fayet G, Lissitzky S. Thyrotropin-binding properties of isolated thyroid cells and their purified plasma membranes. Relation of thyrotropin-specific binding to adenylate-cyclase activation. *Eur J Biochem* (1974) 42: 355–65. doi:10.1111/j.1432-1033.1974.tb03347.x
24. Winkler F, Kleinau G, Tarnow P, Rediger A, Grohmann L, Gaetjens I, et al. A new phenotype of nongoitrous and nonautoimmune hyperthyroidism caused by a heterozygous thyrotropin receptor mutation in transmembrane helix 6. *J Clin Endocrinol Metab* (2010) 95:3605–10. doi:10.1210/jc.2010-0112
25. Kohn LD, Saji M, Akamizu T, Ikuyama S, Isozaki O, Kohn AD, et al. Receptors of the thyroid: the thyrotropin receptor is only the first violinist of a symphony orchestra. *Adv Exp Med Biol* (1989) 261:151–209. doi:10.1007/978-1-4757-2058-7_7
26. Vassart G, Kleinau G. TSH receptor mutations and diseases. In: De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM, et al., editors. *Endotext*. South Dartmouth, MA: MDTEXT.COM, INC. (2014). Available from: www.endotext.org
27. Bolonkin D, Tate RL, Luber JH, Kohn LD, Winand RJ. Experimental exophthalmos. Binding of thyrotropin and an exophthalmogenic factor derived from thyrotropin to retro-orbital tissue plasma membranes. *J Biol Chem* (1975) 250:6516–21.
28. Dobyns BM. Studies on exopthalmos produced by thyrotropic hormone; changes induced in various tissues and organs (including the orbit) by thyrotropic hormone and their relationship to exophthalmos. *Surg Gynecol Obstet* (1946) 82:609–18.
29. Fernando R, Atkins S, Raychaudhuri N, Lu Y, Li B, Douglas RS, et al. Human fibrocytes coexpress thyroglobulin and thyrotropin receptor. *Proc Natl Acad Sci U S A* (2012) 109:7427–32. doi:10.1073/pnas.1202064109
30. Stan MN, Bahn RS. Risk factors for development or deterioration of Graves' ophthalmopathy. *Thyroid* (2010) 20:777–83. doi:10.1089/thy.2010.1634
31. Wiersinga WM. Autoimmunity in Graves' ophthalmopathy: the result of an unfortunate marriage between TSH receptors and IGF-1 receptors? *J Clin Endocrinol Metab* (2011) 96:2386–94. doi:10.1210/jc.2011-0307
32. Smith BR, Hall R. Binding of thyroid stimulators to thyroid membranes. *FEBS Lett* (1974) 42:301–4. doi:10.1016/0014-5793(74)80751-9
33. Wolff J, Winand RJ, Kohn LD. The contribution of subunits of thyroid stimulating hormone to the binding and biological activity of thyrotropin. *Proc Natl Acad Sci U S A* (1974) 71:3460–4. doi:10.1073/pnas.71.9.3460
34. Davies TF, Ando T, Lin RY, Tomer Y, Latif R. Thyrotropin receptor-associated diseases: from adenomata to Graves disease. *J Clin Invest* (2005) 115:1972–83. doi:10.1172/JCI26031
35. Dumont JE, Lamy F, Roger P, Maenhaut C. Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors. *Physiol Rev* (1992) 72:667–97.
36. Kleinau G, Neumann S, Gruters A, Krude H, Biebermann H. Novel insights on thyroid-stimulating hormone receptor signal transduction. *Endocr Rev* (2013) 34:691–724. doi:10.1210/er.2012-1072
37. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM. The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. *Endocr Rev* (1998) 19:673–716. doi:10.1210/edrv.19.6.0352
38. Szkudlinski MW, Fremont V, Ronin C, Weintraub BD. Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. *Physiol Rev* (2002) 82:473–502. doi:10.1152/physrev.00031.2001
39. Gershengorn MC, Neumann S. Update in TSH receptor agonists and antagonists. *J Clin Endocrinol Metab* (2012) 97:4287–92. doi:10.1210/jc.2012-3080
40. Latif R, Ali MR, Ma R, David M, Morshed SA, Ohlmeyer M, et al. New small molecule agonists to the thyrotropin receptor. *Thyroid* (2015) 25:51–62. doi:10.1089/thy.2014.0119
41. Kleinau G, Brehm M, Wiedemann U, Labudde D, Leser U, Krause G. Implications for molecular mechanisms of glycoprotein hormone receptors using a new sequence-structure-function analysis resource. *Mol Endocrinol* (2007) 21:574–80. doi:10.1210/me.2006-0309
42. Kleinau G, Kreuchwig A, Worth CL, Krause G. An interactive web-tool for molecular analyses links naturally occurring mutation data with three-dimensional structures of the rhodopsin-like glycoprotein hormone receptors. *Hum Mutat* (2010) 31:E1519–25. doi:10.1002/humu.21265
43. Kreuchwig A, Kleinau G, Krause G. Research resource: novel structural insights bridge gaps in glycoprotein hormone receptor analyses. *Mol Endocrinol* (2013) 27:1357–63. doi:10.1210/me.2013-1115
44. Kreuchwig A, Kleinau G, Kreuchwig F, Worth CL, Krause G. Research resource: update and extension of a glycoprotein hormone receptors web application. *Mol Endocrinol* (2011) 25:707–12. doi:10.1210/me.2010-0510
45. Latif R, Realubit RB, Karan C, Mezei M, Davies TF. TSH receptor signaling abrogation by a novel small molecule. *Front Endocrinol* (2016) 7:130. doi:10.3389/fendo.2016.00130
46. Neumann S, Eliseeva E, McCoy JG, Napolitano G, Giuliani C, Monaco F, et al. A new small-molecule antagonist inhibits Graves' disease antibody activation of the TSH receptor. *J Clin Endocrinol Metab* (2011) 96:548–54. doi:10.1210/jc.2010-1935
47. Neumann S, Huang W, Eliseeva E, Titus S, Thomas CJ, Gershengorn MC. A small molecule inverse agonist for the human thyroid-stimulating hormone receptor. *Endocrinology* (2010) 151:3454–9. doi:10.1210/en.2010-0199
48. Neumann S, Huang W, Titus S, Krause G, Kleinau G, Alberobello AT, et al. Small-molecule agonists for the thyrotropin receptor stimulate thyroid function in human thyrocytes and mice. *Proc Natl Acad Sci U S A* (2009) 106:12471–6. doi:10.1073/pnas.0904506106
49. Neumann S, Kleinau G, Costanzi S, Moore S, Jiang JK, Raaka BM, et al. A low-molecular-weight antagonist for the human thyrotropin receptor with therapeutic potential for hyperthyroidism. *Endocrinology* (2008) 149(12):5945–50. doi:10.1210/en.2008-0836
50. Neumann S, Nir EA, Eliseeva E, Huang W, Marugan J, Xiao J, et al. A selective TSH receptor antagonist inhibits stimulation of thyroid function in female mice. *Endocrinology* (2014) 155(1):310–4. doi:10.1210/en.2013-1835
51. Neumann S, Pope A, Geras-Raaka E, Raaka BM, Bahn RS, Gershengorn MC, et al. antagonist inhibits thyrotropin receptor-mediated stimulation of cAMP production in Graves' orbital fibroblasts. *Thyroid* (2012) 22:839–43. doi:10.1089/thy.2011.0520
52. Neumann S, Raaka BM, Gershengorn MC. Human TSH receptor ligands as pharmacological probes with potential clinical application. *Expert Rev Endocrinol Metab* (2009) 4:669. doi:10.1586/eem.09.36
53. Kleinau G, Mueller S, Jaeschke H, Grzesik P, Neumann S, Diehl A, et al. Defining structural and functional dimensions of the extracellular thyrotropin receptor region. *J Biol Chem* (2011) 286:22622–31. doi:10.1074/jbc.M110.211193
54. Caltabiano G, Campillo M, De Leener A, Smits G, Vassart G, Costagliola S, et al. The specificity of binding of glycoprotein hormones to their receptors. *Cell Mol Life Sci* (2008) 65:2484–92. doi:10.1007/s00018-008-8002-9
55. Chen CR, McLachlan SM, Rapoport B. Thyrotropin (TSH) receptor residue E251 in the extracellular leucine-rich repeat domain is critical for linking TSH binding to receptor activation. *Endocrinology* (2010) 151:1940–7. doi:10.1210/en.2009-1430
56. Sanders J, Chirgadze DY, Sanders P, Baker S, Sullivan A, Bhardwaja A, et al. Crystal structure of the TSH receptor in complex with a thyroid-stimulating autoantibody. *Thyroid* (2007) 17:395–410. doi:10.1089/thy.2007.0041

57. Sanders P, Young S, Sanders J, Kabelis K, Baker S, Sullivan A, et al. Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. *J Mol Endocrinol* (2011) 46:81–99. doi:10.1530/JME-10-0127
58. Smits G, Campillo M, Govaerts C, Janssens V, Richter C, Vassart G, et al. Glycoprotein hormone receptors: determinants in leucine-rich repeats responsible for ligand specificity. *EMBO J* (2003) 22:2692–703. doi:10.1093/emboj/cdg260
59. Nagayama Y, Nishihara E, Namba H, Yamashita S, Niwa M. Identification of the sites of asparagine-linked glycosylation on the human thyrotropin receptor and studies on their role in receptor function and expression. *J Pharmacol Exp Ther* (2000) 295:404–9.
60. Nunez Miguel R, Sanders J, Furmaniak J, Rees Smith B. Glycosylation pattern analysis of glycoprotein hormones and their receptors. *J Mol Endocrinol* (2017) 58(1):25–41. doi:10.1530/JME-16-0169
61. Oda Y, Sanders J, Roberts S, Maruyama M, Kiddie A, Furmaniak J, et al. Analysis of carbohydrate residues on recombinant human thyrotropin receptor. *J Clin Endocrinol Metab* (1999) 84:2119–25. doi:10.1210/jcem.84.6.5756
62. Russo D, Chazenbalk GD, Nagayama Y, Wadsworth HL, Rapoport B. Site-directed mutagenesis of the human thyrotropin receptor: role of asparagine-linked oligosaccharides in the expression of a functional receptor. *Mol Endocrinol* (1991) 5:29–33. doi:10.1210/mend-5-1-29
63. Kleinau G, Krause G. Thyrotropin and homologous glycoprotein hormone receptors: structural and functional aspects of extracellular signaling mechanisms. *Endocr Rev* (2009) 30:133–51. doi:10.1210/er.2008-0044
64. Fan QR, Hendrickson WA. Structure of human follicle-stimulating hormone in complex with its receptor. *Nature* (2005) 433:269–77. doi:10.1038/nature03206
65. Jiang X, Liu H, Chen X, Chen PH, Fischer D, Sriraman V, et al. Structure of follicle-stimulating hormone in complex with the entire ectodomain of its receptor. *Proc Natl Acad Sci U S A* (2012) 109:12491–6. doi:10.1073/pnas.1206643109
66. Enkhbayar P, Kamiya M, Osaki M, Matsumoto T, Matsushima N. Structural principles of leucine-rich repeat (LRR) proteins. *Proteins* (2004) 54:394–403. doi:10.1002/prot.10605
67. Kobe B, Deisenhofer J. A structural basis of the interactions between leucine-rich repeats and protein ligands. *Nature* (1995) 374:183–6. doi:10.1038/374183a0
68. Matsushima N, Tachi N, Kuroki Y, Enkhbayar P, Osaki M, Kamiya M, et al. Structural analysis of leucine-rich-repeat variants in proteins associated with human diseases. *Cell Mol Life Sci* (2005) 62:2771–91. doi:10.1007/s00018-005-5187-z
69. Ho SC, Van Sande J, Lefort A, Vassart G, Costagliola S. Effects of mutations involving the highly conserved S281HCC motif in the extracellular domain of the thyrotropin (TSH) receptor on TSH binding and constitutive activity. *Endocrinology* (2001) 142:2760–7. doi:10.1210/en.142.7.2760
70. Duprez L, Parma J, Costagliola S, Hermans J, Van Sande J, Dumont JE, et al. Constitutive activation of the TSH receptor by spontaneous mutations affecting the N-terminal extracellular domain. *FEBS Lett* (1997) 409:469–74. doi:10.1016/S0014-5793(97)00532-2
71. Kopp P, Muirhead S, Jourdain N, Gu WX, Jameson JL, Rodd C. Congenital hyperthyroidism caused by a solitary toxic adenoma harboring a novel somatic mutation (serine281 – >isoleucine) in the extracellular domain of the thyrotropin receptor. *J Clin Invest* (1997) 100:1634–9. doi:10.1172/JCI119687
72. Ho SC, Goh SS, Li S, Khoo DH, Paterson M. Effects of mutations involving cysteine residues distal to the S281HCC motif at the C-terminus on the functional characteristics of a truncated ectodomain-only thyrotropin receptor anchored on glycosylphosphatidyl-inositol. *Thyroid* (2008) 18:1313–9. doi:10.1089/thy.2008.0240
73. Jaeschke H, Neumann S, Kleinau G, Mueller S, Claus M, Krause G, et al. An aromatic environment in the vicinity of serine 281 is a structural requirement for thyrotropin receptor function. *Endocrinology* (2006) 147:1753–60. doi:10.1210/en.2005-1138
74. Rodien P, Bremont C, Sanson ML, Parma J, Van Sande J, Costagliola S, et al. Familial gestational hyperthyroidism caused by a mutant thyrotropin receptor hypersensitive to human chorionic gonadotropin. *N Engl J Med* (1998) 339:1823–6. doi:10.1056/NEJM199812173392505
75. Smits G, Govaerts C, Nubourgh I, Pardo L, Vassart G, Costagliola S. Lysine 183 and glutamic acid 157 of the TSH receptor: two interacting residues with a key role in determining specificity toward TSH and human CG. *Mol Endocrinol* (2002) 16:722–35. doi:10.1210/mend.16.4.0815
76. Costagliola S, Panneels V, Bonomi M, Koch J, Many MC, Smits G, et al. Tyrosine sulfation is required for agonist recognition by glycoprotein hormone receptors. *EMBO J* (2002) 21:504–13. doi:10.1093/emboj/21.4.504
77. Jiang X, Dias JA, He X. Structural biology of glycoprotein hormones and their receptors: insights to signaling. *Mol Cell Endocrinol* (2014) 382:424–51. doi:10.1016/j.mce.2013.08.021
78. Chazenbalk GD, Tanaka K, Nagayama Y, Kakinuma A, Jaume JC, McLachlan SM, et al. Evidence that the thyrotropin receptor ectodomain contains not one, but two, cleavage sites. *Endocrinology* (1997) 138:2893–9. doi:10.1210/en.138.7.2893
79. Rapoport B, McLachlan SM. TSH receptor cleavage into subunits and shedding of the A-subunit; a molecular and clinical perspective. *Endocr Rev* (2016) 2016:23–42. doi:10.1210/er.2015-1098.2016.1.test
80. Couet J, de Bernard S, Loosfelt H, Saunier B, Milgrom E, Misrahi M. Cell surface protein disulfide-isomerase is involved in the shedding of human thyrotropin receptor ectodomain. *Biochemistry* (1996) 35:14800–5. doi:10.1021/bi961359w
81. Loosfelt H, Pichon C, Jolivet A, Misrahi M, Caillou B, Jamous M, et al. Two-subunit structure of the human thyrotropin receptor. *Proc Natl Acad Sci U S A* (1992) 89:3765–9. doi:10.1073/pnas.89.9.3765
82. Misrahi M, Milgrom E. Cleavage and shedding of the TSH receptor. *Eur J Endocrinol* (1997) 137:599–602. doi:10.1530/eje.0.1370599
83. Quellari M, Desroches A, Beau I, Beaudeux E, Misrahi M. Role of cleavage and shedding in human thyrotropin receptor function and trafficking. *Eur J Biochem* (2003) 270:3486–97. doi:10.1046/j.1432-1033.2003.03718.x
84. Vassart G, Costagliola S. A physiological role for the posttranslational cleavage of the thyrotropin receptor? *Endocrinology* (2004) 145:1–3. doi:10.1210/en.2003-1225
85. Mizutori Y, Chen CR, Latrofa F, McLachlan SM, Rapoport B. Evidence that shed thyrotropin receptor A subunits drive affinity maturation of autoantibodies causing Graves' disease. *J Clin Endocrinol Metab* (2009) 94:927–35. doi:10.1210/jc.2008-2134
86. Rapoport B, Aliesky HA, Chen CR, McLachlan SM. Evidence that TSH receptor A-subunit multimers, not monomers, drive antibody affinity maturation in Graves' disease. *J Clin Endocrinol Metab* (2015) 100:E871–5. doi:10.1210/jc.2015-1528
87. Chazenbalk GD, Chen CR, McLachlan SM, Rapoport B. Does thyrotropin cleave its cognate receptor? *Endocrinology* (2004) 145:4–10. doi:10.1210/en.2003-1002
88. Kaczur V, Puskas LG, Nagy ZU, Miled N, Rebai A, Juhasz F, et al. Cleavage of the human thyrotropin receptor by ADAM10 is regulated by thyrotropin. *J Mol Recognit* (2007) 20:392–404. doi:10.1002/jmr.851
89. Vu MT, Radu A, Ghinea N. The cleavage of thyroid-stimulating hormone receptor is dependent on cell-cell contacts and regulates the hormonal stimulation of phospholipase c. *J Cell Mol Med* (2009) 13:2253–60. doi:10.1111/j.1582-4934.2008.00422.x
90. Bruser A, Schulz A, Rothemund S, Ricken A, Calebiro D, Kleinau G, et al. The activation mechanism of glycoprotein hormone receptors with implications in the cause and therapy of endocrine diseases. *J Biol Chem* (2016) 291:508–20. doi:10.1074/jbc.M115.701102
91. Krause G, Kreuchwig A, Kleinau G. Extended and structurally supported insights into extracellular hormone binding, signal transduction and organization of the thyrotropin receptor. *PLoS One* (2012) 7:e52920. doi:10.1371/journal.pone.0052920
92. Bonomi M, Busnelli M, Persani L, Vassart G, Costagliola S. Structural differences in the hinge region of the glycoprotein hormone receptors: evidence from the sulfated tyrosine residues. *Mol Endocrinol* (2006) 20:3351–63. doi:10.1210/me.2005-0521
93. Bruysters M, Verhoef-Post M, Themmen AP. Asp330 and Tyr331 in the C-terminal cysteine-rich region of the luteinizing hormone receptor are key residues in hormone-induced receptor activation. *J Biol Chem* (2008) 283:25821–8. doi:10.1074/jbc.M804395200
94. Grzesik P, Kreuchwig A, Rutz C, Furkert J, Wiesner B, Schuelein R, et al. Differences in signal activation by LH and hCG are mediated by the LH/CG receptor's extracellular hinge region. *Front Endocrinol* (2015) 6:140. doi:10.3389/fendo.2015.00140

95. Grzesik P, Teichmann A, Furkert J, Rutz C, Wiesner B, Kleinau G, et al. Differences between lutropin-mediated and choriogonadotropin-mediated receptor activation. *FEBS J* (2014) 281:1479–92. doi:10.1111/febs.12718
96. Deupi X, Kobilka B. Activation of G protein-coupled receptors. *Adv Protein Chem* (2007) 74:137–66. doi:10.1016/S0065-3233(07)74004-4
97. Deupi X, Standfuss J. Structural insights into agonist-induced activation of G-protein-coupled receptors. *Curr Opin Struct Biol* (2011) 21:541–51. doi:10.1016/j.sbi.2011.06.002
98. Hanson MA, Stevens RC. Discovery of new GPCR biology: one receptor structure at a time. *Structure* (2009) 17:8–14. doi:10.1016/j.str.2008.12.003
99. Katritch V, Cherezov V, Stevens RC. Diversity and modularity of G protein-coupled receptor structures. *Trends Pharmacol Sci* (2012) 33:17–27. doi:10.1016/j.tips.2011.09.003
100. Costanzi S. Homology modeling of class a G protein-coupled receptors. *Methods Mol Biol* (2012) 857:259–79. doi:10.1007/978-1-61779-588-6_11
101. Costanzi S. Modeling G protein-coupled receptors and their interactions with ligands. *Curr Opin Struct Biol* (2013) 23:185–90. doi:10.1016/j.sbi.2013.01.008
102. Costanzi S, Wang K. The GPCR crystallography boom: providing an invaluable source of structural information and expanding the scope of homology modeling. *Adv Exp Med Biol* (2014) 796:3–13. doi:10.1007/978-94-007-7423-0_1
103. Worth CL, Kleinau G, Krause G. Comparative sequence and structural analyses of G-protein-coupled receptor crystal structures and implications for molecular models. *PLoS One* (2009) 4:e7011. doi:10.1371/journal.pone.0007011
104. Chantreau V, Taddese B, Munier M, Gourdin L, Henrion D, Rodien P, et al. Molecular insights into the transmembrane domain of the thyrotropin receptor. *PLoS One* (2015) 10:e0142250. doi:10.1371/journal.pone.0142250
105. Kleinau G, Claus M, Jaeschke H, Mueller S, Neumann S, Paschke R, et al. Contacts between extracellular loop two and transmembrane helix six determine basal activity of the thyroid-stimulating hormone receptor. *J Biol Chem* (2007) 282:518–25. doi:10.1074/jbc.M606176200
106. Kleinau G, Haas AK, Neumann S, Worth CL, Hoyer I, Furkert J, et al. Signaling-sensitive amino acids surround the allosteric ligand binding site of the thyrotropin receptor. *FASEB J* (2010) 24:2347–54. doi:10.1096/fj.09-149146
107. Urizar E, Claeysen S, Deupi X, Govaerts C, Costagliola S, Vassart G, et al. An activation switch in the rhodopsin family of G protein-coupled receptors: the thyrotropin receptor. *J Biol Chem* (2005) 280:17135–41. doi:10.1074/jbc.M414678200
108. Labadi A, Grassi ES, Gellen B, Kleinau G, Biebermann H, Ruzsa B, et al. Loss-of-function variants in a Hungarian cohort reveal structural insights on TSH receptor maturation and signaling. *J Clin Endocrinol Metab* (2015) 100:E1039–45. doi:10.1210/jc.2014-4511
109. Ringkananont U, Van Durme J, Montanelli L, Ugrasbul F, Yu YM, Weiss RE, et al. Repulsive separation of the cytoplasmic ends of transmembrane helices 3 and 6 is linked to receptor activation in a novel thyrotropin receptor mutant (M626I). *Mol Endocrinol* (2006) 20:893–903. doi:10.1210/me.2005-0339
110. Moore S, Jaeschke H, Kleinau G, Neumann S, Costanzi S, Jiang JK, et al. Evaluation of small-molecule modulators of the luteinizing hormone/choriogonadotropin and thyroid stimulating hormone receptors: structure-activity relationships and selective binding patterns. *J Med Chem* (2006) 49:3888–96. doi:10.1021/jm060247s
111. Kleinau G, Jaeschke H, Worth CL, Mueller S, Gonzalez J, Paschke R, et al. Principles and determinants of G-protein coupling by the rhodopsin-like thyrotropin receptor. *PLoS One* (2010) 5:e9745. doi:10.1371/journal.pone.0009745
112. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* (2000) 289:739–45. doi:10.1126/science.289.5480.739
113. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, et al. High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* (2007) 318:1258–65. doi:10.1126/science.1150577
114. Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* (2007) 450:383–7. doi:10.1038/nature06325
115. Park JH, Scheerer P, Hofmann KP, Choe HW, Ernst OP. Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature* (2008) 454:183–7. doi:10.1038/nature07063
116. Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, et al. Crystal structure of opsin in its G-protein-interacting conformation. *Nature* (2008) 455:497–502. doi:10.1038/nature07330
117. Choe HW, Kim YJ, Park JH, Morizumi T, Pai EF, Krauss N, et al. Crystal structure of metarhodopsin II. *Nature* (2011) 471:651–5. doi:10.1038/nature09789
118. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* (2011) 477:549–55. doi:10.1038/nature10361
119. Carpenter B, Nehme R, Warne T, Leslie AG, Tate CG. Structure of the adenosine A(2A) receptor bound to an engineered G protein. *Nature* (2016) 536:104–7. doi:10.1038/nature18966
120. Kleinau G, Jaeschke H, Mueller S, Worth CL, Paschke R, Krause G. Molecular and structural effects of inverse agonistic mutations on signaling of the thyrotropin receptor – a basally active GPCR. *Cell Mol Life Sci* (2008) 65:3664–76. doi:10.1007/s00018-008-8450-2
121. Isberg V, Mordalski S, Munk C, Rataj K, Harpsoe K, Hauser AS, et al. GPCRdb: an information system for G protein-coupled receptors. *Nucleic Acids Res* (2016) 44:D356–64. doi:10.1093/nar/gkv1178
122. Munk C, Isberg V, Mordalski S, Harpsoe K, Rataj K, Hauser AS, et al. GPCRdb: the G protein-coupled receptor database – an introduction. *Br J Pharmacol* (2016) 173:2195–207. doi:10.1111/bph.13509
123. Worth CL, Kreuchwig A, Kleinau G, Krause G. GPCR-SSFE: a comprehensive database of G-protein-coupled receptor template predictions and homology models. *BMC Bioinformatics* (2011) 12:185. doi:10.1186/1471-2105-12-185
124. Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relationships in G-protein coupled receptors. *Methods Neurosci* (1995) 25:366–428. doi:10.1016/S1043-9471(05)80049-7
125. Isberg V, de Graaf C, Bortolato A, Cherezov V, Katritch V, Marshall FH, et al. Generic GPCR residue numbers – aligning topology maps while minding the gaps. *Trends Pharmacol Sci* (2015) 36:22–31. doi:10.1016/j.tips.2014.11.001
126. Kleinau G, Hoyer I, Kreuchwig A, Haas AK, Rutz C, Furkert J, et al. From molecular details of the interplay between transmembrane helices of the thyrotropin receptor to general aspects of signal transduction in family A G-protein-coupled receptors (GPCRs). *J Biol Chem* (2011) 286:25859–71. doi:10.1074/jbc.M110.196980
127. Hanson MA, Roth CB, Jo E, Griffith MT, Scott FL, Reinhart G, et al. Crystal structure of a lipid G protein-coupled receptor. *Science* (2012) 335:851–5. doi:10.1126/science.1215904
128. Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, et al. Structure of the human P2Y12 receptor in complex with an antithrombotic drug. *Nature* (2014) 509:115–8. doi:10.1038/nature13288
129. Chrencik JE, Roth CB, Terakado M, Kurata H, Omi R, Kihara Y, et al. Crystal structure of antagonist bound human lysophosphatidic acid receptor 1. *Cell* (2015) 161:1633–43. doi:10.1016/j.cell.2015.06.002
130. Kleinau G, Biebermann H. Constitutive activities in the thyrotropin receptor: regulation and significance. *Adv Pharmacol* (2014) 70:81–119. doi:10.1016/B978-0-12-417197-8.00003-1
131. Nunez Miguel R, Sanders J, Furmaniak J, Smith BR. Structure and activation of the TSH receptor transmembrane domain. *Auto Immun Highlights* (2017) 8:2. doi:10.1007/s13317-016-0090-1
132. Sali A, Blundell TL. Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* (1993) 234:779–815. doi:10.1006/jmbi.1993.1626
133. Ismer J, Rose AS, Tiemann JK, Goede A, Preissner R, Hildebrand PW. SL2: an interactive webtool for modeling of missing segments in proteins. *Nucleic Acids Res* (2016) 44:W390–4. doi:10.1093/nar/gkw297
134. Ali MR, Latif R, Davies TF, Mezei M. Monte Carlo loop refinement and virtual screening of the thyroid-stimulating hormone receptor transmembrane domain. *J Biomol Struct Dyn* (2015) 33:1140–52. doi:10.1080/07391102.2014.932310
135. Schaarschmidt J, Nagel MB, Huth S, Jaeschke H, Moretti R, Hintze V, et al. Rearrangement of the extracellular domain/extracellular loop 1 interface is critical for thyrotropin receptor activation. *J Biol Chem* (2016) 291:14095–108. doi:10.1074/jbc.M115.709659

136. Hofmann KP, Scheerer P, Hildebrand PW, Choe HW, Park JH, Heck M, et al. A G protein-coupled receptor at work: the rhodopsin model. *Trends Biochem Sci* (2009) 34:540–52. doi:10.1016/j.tibs.2009.07.005
137. Manglik A, Kobilka B. The role of protein dynamics in GPCR function: insights from the beta2AR and rhodopsin. *Curr Opin Cell Biol* (2014) 27: 136–43. doi:10.1016/j.ceb.2014.01.008
138. Fredriksson R, Schioth HB. The repertoire of G-protein-coupled receptors in fully sequenced genomes. *Mol Pharmacol* (2005) 67:1414–25. doi:10.1124/mol.104.009001
139. Strotmann R, Schrock K, Boselt I, Staubert C, Russ A, Schoneberg T. Evolution of GPCR: change and continuity. *Mol Cell Endocrinol* (2011) 331:170–8. doi:10.1016/j.mce.2010.07.012
140. Kimple AJ, Bosch DE, Giguere PM, Siderovski DP. Regulators of G-protein signaling and their Galpha substrates: promises and challenges in their use as drug discovery targets. *Pharmacol Rev* (2011) 63:728–49. doi:10.1124/pr.110.003038
141. O'Hayre M, Vazquez-Prado J, Kufareva I, Stawiski EW, Handel TM, Seshagiri S, et al. The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat Rev Cancer* (2013) 13:412–24. doi:10.1038/nrc3521
142. Plagge A, Kelsey G, Germain-Lee EL. Physiological functions of the imprinted Gnas locus and its protein variants Galpha(s) and XLalpha(s) in human and mouse. *J Endocrinol* (2008) 196:193–214. doi:10.1677/JOE-07-0544
143. Kleinau G, Kalveram L, Kohrle J, Szkudlinski M, Schomburg L, Biebermann H, et al. Minireview: insights into the structural and molecular consequences of the TSH-beta mutation C105Vfs114X. *Mol Endocrinol* (2016) 30:954–64. doi:10.1210/me.2016-1065
144. Schoenmakers N, Alatzoglou KS, Chatterjee VK, Dattani MT. Recent advances in central congenital hypothyroidism. *J Endocrinol* (2015) 227: R51–71. doi:10.1530/JOE-15-0341
145. Fox KM, Dias JA, Van Roey P. Three-dimensional structure of human follicle-stimulating hormone. *Mol Endocrinol* (2001) 15:378–89. doi:10.1210/mend.15.3.0603
146. Wu H, Lustbader JW, Liu Y, Canfield RE, Hendrickson WA. Structure of human chorionic gonadotropin at 2.6 A resolution from MAD analysis of the selenomethionyl protein. *Structure* (1994) 2:545–58. doi:10.1016/S0969-2126(00)00054-X
147. Lapthorn AJ, Harris DC, Littlejohn A, Lustbader JW, Canfield RE, Machin KJ, et al. Crystal structure of human chorionic gonadotropin. *Nature* (1994) 369:455–61. doi:10.1038/369455a0
148. Tegoni M, Spinelli S, Verhoeyen M, Davis P, Cambillau C. Crystal structure of a ternary complex between human chorionic gonadotropin (hCG) and two Fv fragments specific for the alpha and beta-subunits. *J Mol Biol* (1999) 289:1375–85. doi:10.1006/jmbi.1999.2845
149. Chen CR, Hubbard PA, Salazar LM, McLachlan SM, Murali R, Rapoport B. Crystal structure of a TSH receptor monoclonal antibody: insight into Graves' disease pathogenesis. *Mol Endocrinol* (2015) 29:99–107. doi:10.1210/me.2014-1257
150. Graves PN, Vlase H, Bobovnikova Y, Davies TF. Multimeric complex formation by the thyrotropin receptor in solubilized thyroid membranes. *Endocrinology* (1996) 137:3915–20. doi:10.1210/en.137.9.3915
151. Graves PN, Vlase H, Davies TF. Folding of the recombinant human thyrotropin (TSH) receptor extracellular domain: identification of folded monomeric and tetrameric complexes that bind TSH receptor autoantibodies. *Endocrinology* (1995) 136:521–7. doi:10.1210/en.136.2.521
152. Manglik A, Kruse AC, Kobilka TS, Thian FS, Mathiesen JM, Sunahara RK, et al. Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature* (2012) 485:321–6. doi:10.1038/nature10954
153. Wu H, Wacker D, Mileni M, Katritch V, Han GW, Vardy E, et al. Structure of the human kappa-opioid receptor in complex with JDTic. *Nature* (2012) 485:327–32. doi:10.1038/nature10939
154. Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, et al. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* (2010) 330:1066–71. doi:10.1126/science.1194396
155. Huang J, Chen S, Zhang JJ, Huang XY. Crystal structure of oligomeric beta1-adrenergic G protein-coupled receptors in ligand-free basal state. *Nat Struct Mol Biol* (2013) 20:419–25. doi:10.1038/nsmb.2504
156. Coke CJ, Scarlett KA, Chetram MA, Jones KJ, Sandifer BJ, Davis AS, et al. Simultaneous activation of induced heterodimerization between CXCR4 chemokine receptor and cannabinoid receptor 2 (CB2) reveals a mechanism for regulation of tumor progression. *J Biol Chem* (2016) 291:9991–10005. doi:10.1074/jbc.M115.712661
157. Gaitonde SA, Gonzalez-Maeso J. Contribution of heteromerization to G protein-coupled receptor function. *Curr Opin Pharmacol* (2016) 32:23–31. doi:10.1016/j.coph.2016.10.006
158. Liu H, Tian Y, Ji B, Lu H, Xin Q, Jiang Y, et al. Heterodimerization of the kappa opioid receptor and neurotensin receptor 1 contributes to a novel beta-arrestin-2-biased pathway. *Biochim Biophys Acta* (2016) 1863:2719–38. doi:10.1016/j.bbamcr.2016.07.009
159. Rozenfeld R, Devi LA. Receptor heteromerization and drug discovery. *Trends Pharmacol Sci* (2010) 31:124–30. doi:10.1016/j.tips.2009.11.008
160. Rozenfeld R, Devi LA. Exploring a role for heteromerization in GPCR signalling specificity. *Biochem J* (2011) 433:11–8. doi:10.1042/BJ20100458
161. Petryszak R, Keays M, Tang YA, Fonseca NA, Barrera E, Burdett T, et al. Expression atlas update – an integrated database of gene and protein expression in humans, animals and plants. *Nucleic Acids Res* (2016) 44:D746–52. doi:10.1093/nar/gkv1045
162. Kang Y, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, et al. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* (2015) 523:561–7. doi:10.1038/nature14656
163. Zhou XE, Gao X, Barty A, Kang Y, He Y, Liu W, et al. X-ray laser diffraction for structure determination of the rhodopsin-arrestin complex. *Sci Data* (2016) 3:160021. doi:10.1038/sdata.2016.21
164. Szczepek M, Beyriere F, Hofmann KP, Elgeti M, Kazmin R, Rose A, et al. Crystal structure of a common GPCR-binding interface for G protein and arrestin. *Nat Commun* (2014) 5:4801. doi:10.1038/ncomms5801
165. Zhan X, Gimenez LE, Gurevich VV, Spiller BW. Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes. *J Mol Biol* (2011) 406:467–78. doi:10.1016/j.jmb.2010.12.034
166. Kim YJ, Hofmann KP, Ernst OP, Scheerer P, Choe HW, Sommer ME. Crystal structure of pre-activated arrestin p44. *Nature* (2013) 497:142–6. doi:10.1038/nature12133
167. Shukla AK, Manglik A, Kruse AC, Xiao K, Reis RI, Tseng WC, et al. Structure of active beta-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature* (2013) 497:137–41. doi:10.1038/nature12120
168. Coleman DE, Berghuis AM, Lee E, Linder ME, Gilman AG, Sprang SR. Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. *Science* (1994) 265:1405–12. doi:10.1126/science.8073283
169. Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG, et al. The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. *Cell* (1995) 83:1047–58. doi:10.1016/0092-8674(95)90220-1
170. Sunahara RK, Tesmer JJ, Gilman AG, Sprang SR. Crystal structure of the adenylyl cyclase activator Gsalpha. *Science* (1997) 278:1943–7. doi:10.1126/science.278.5345.1943
171. Nishimura A, Kitano K, Takasaki J, Taniguchi M, Mizuno N, Tago K, et al. Structural basis for the specific inhibition of heterotrimeric Gq protein by a small molecule. *Proc Natl Acad Sci U S A* (2010) 107:13666–71. doi:10.1073/pnas.1003553107
172. Waldo GL, Ricks TK, Hicks SN, Cheever ML, Kawano T, Tsuboi K, et al. Kinetic scaffolding mediated by a phospholipase C-beta and Gq signaling complex. *Science* (2010) 330:974–80. doi:10.1126/science.1193438
173. Tesmer VM, Kawano T, Shankaranarayanan A, Kozasa T, Tesmer JJ. Snapshot of activated G proteins at the membrane: the Galphaq-GRK2-Gbetagamma complex. *Science* (2005) 310:1686–90. doi:10.1126/science.1118890
174. Sower SA, Decatur WA, Hausken KN, Marquis TJ, Barton SL, Gargan J, et al. Emergence of an ancestral glycoprotein hormone in the pituitary of the sea lamprey, a basal vertebrate. *Endocrinology* (2015) 156:3026–37. doi:10.1210/en.2014-1797
175. Nakabayashi K, Matsumi H, Bhalla A, Bae J, Mosselman S, Hsu SY, et al. Thyrostimulin, a heterodimer of two new human glycoprotein hormone subunits, activates the thyroid-stimulating hormone receptor. *J Clin Invest* (2002) 109:1445–52. doi:10.1172/JCI0214340
176. Okada SL, Ellsworth JL, Durnam DM, Haugen HS, Holloway JL, Kelley ML, et al. A glycoprotein hormone expressed in corticotrophs exhibits unique

binding properties on thyroid-stimulating hormone receptor. *Mol Endocrinol* (2006) 20:414–25. doi:10.1210/me.2005-0270

177. Jaschke H, Neumann S, Moore S, Thomas CJ, Colson AO, Costanzi S, et al. A low molecular weight agonist signals by binding to the transmembrane domain of thyroid-stimulating hormone receptor (TSHR) and luteinizing hormone/chorionic gonadotropin receptor (LHCGR). *J Biol Chem* (2006) 281:9841–4. doi:10.1074/jbc.C600014200
178. Boutin A, Eliseeva E, Gershengorn MC, Neumann S. beta-Arrestin-1 mediates thyrotropin-enhanced osteoblast differentiation. *FASEB J* (2014) 28:3446–55. doi:10.1096/fj.14-251124
179. Frenzel R, Voigt C, Paschke R. The human thyrotropin receptor is predominantly internalized by beta-arrestin 2. *Endocrinology* (2006) 147:3114–22. doi:10.1210/en.2005-0687
180. Neumann S, Geras-Raaka E, Marcus-Samuels B, Gershengorn MC. Persistent cAMP signaling by thyrotropin (TSH) receptors is not dependent on internalization. *FASEB J* (2010) 24:3992–9. doi:10.1096/fj.10-161745
181. Werthmann RC, Volpe S, Lohse MJ, Calebiro D. Persistent cAMP signaling by internalized TSH receptors occurs in thyroid but not in HEK293 cells. *FASEB J* (2012) 26:2043–8. doi:10.1096/fj.11-195248
182. Zoenen M, Urizar E, Swillens S, Vassart G, Costagliola S. Evidence for activity-regulated hormone-binding cooperativity across glycoprotein hormone receptor homomers. *Nat Commun* (2012) 3:1007. doi:10.1038/ncomms1991
183. Urizar E, Montanelli L, Loy T, Bonomi M, Swillens S, Gales C, et al. Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. *EMBO J* (2005) 24:1954–64. doi:10.1038/sj.emboj.7600686
184. Latif R, Michalek K, Davies TF. Subunit interactions influence TSHR multimerization. *Mol Endocrinol* (2010) 24:2009–18. doi:10.1210/me.2010-0001
185. Audet M, Bouvier M. Restructuring G-protein-coupled receptor activation. *Cell* (2012) 151:14–23. doi:10.1016/j.cell.2012.09.003
186. Baltoumas FA, Theodoropoulou MC, Hamodrakas SJ. Molecular dynamics simulations and structure-based network analysis reveal structural and functional aspects of G-protein coupled receptor dimer interactions. *J Comput Aided Mol Des* (2016) 30:489–512. doi:10.1007/s10822-016-9919-y
187. Kaczur V, Puskas LG, Takacs M, Racz IA, Szendroi A, Toth S, et al. Evolution of the thyrotropin receptor: a G protein coupled receptor with an intrinsic capacity to dimerize. *Mol Genet Metab* (2003) 78:275–90. doi:10.1016/S1096-7192(03)00036-2
188. Latif R, Graves P, Davies TF. Oligomerization of the human thyrotropin receptor: fluorescent protein-tagged hTSHR reveals post-translational complexes. *J Biol Chem* (2001) 276:45217–24. doi:10.1074/jbc.M103727200
189. Kleinau G, Jaschke H, Neumann S, Lattig J, Paschke R, Krause G. Identification of a novel epitope in the thyroid-stimulating hormone receptor ectodomain acting as intramolecular signaling interface. *J Biol Chem* (2004) 279:51590–600. doi:10.1074/jbc.M404748200
190. Busnelli M, Kleinau G, Muttenthaler M, Stoev S, Manning M, Bibic L, et al. Design and characterization of superpotent bivalent ligands targeting oxytocin receptor dimers via a channel-like structure. *J Med Chem* (2016) 59:7152–66. doi:10.1021/acs.jmedchem.6b00564
191. Tate RL, Schwartz HI, Holmes JM, Kohn LD. Thyrotropin receptors in thyroid plasma membranes. Characteristics of thyrotropin binding and solubilization of thyrotropin receptor activity by tryptic digestion. *J Biol Chem* (1975) 250:6509–15.
192. Mueller S, Kleinau G, Jaeschke H, Paschke R, Krause G. Extended hormone binding site of the human thyroid stimulating hormone receptor: distinctive acidic residues in the hinge region are involved in bovine thyroid stimulating hormone binding and receptor activation. *J Biol Chem* (2008) 283:18048–55. doi:10.1074/jbc.M800449200
193. Mueller S, Kleinau G, Szkudlinski MW, Jaeschke H, Krause G, Paschke R. The superagonistic activity of bovine thyroid-stimulating hormone (TSH) and the human TR1401 TSH analog is determined by specific amino acids in the hinge region of the human TSH receptor. *J Biol Chem* (2009) 284:16317–24. doi:10.1074/jbc.M109.005710
194. Mueller S, Szkudlinski MW, Schaarschmidt J, Gunther R, Paschke R, Jaeschke H. Identification of novel TSH interaction sites by systematic binding analysis of the TSHR hinge region. *Endocrinology* (2011) 152:3268–78. doi:10.1210/en.2011-0153
195. Chen CR, McLachlan SM, Rapoport B. Evidence that the thyroid-stimulating hormone (TSH) receptor transmembrane domain influences kinetics of TSH binding to the receptor ectodomain. *J Biol Chem* (2011) 286:6219–24. doi:10.1074/jbc.M110.211003
196. Chen CR, Salazar LM, McLachlan SM, Rapoport B. The thyrotropin receptor hinge region as a surrogate ligand: identification of loci contributing to the coupling of thyrotropin binding and receptor activation. *Endocrinology* (2012) 153:5058–67. doi:10.1210/en.2012-1376
197. Hamidi S, Chen CR, Mizutori-Sasai Y, McLachlan SM, Rapoport B. Relationship between thyrotropin receptor hinge region proteolytic posttranslational modification and receptor physiological function. *Mol Endocrinol* (2011) 25:184–94. doi:10.1210/me.2010-0401
198. Jaeschke H, Schaarschmidt J, Gunther R, Mueller S. The hinge region of the TSH receptor stabilizes ligand binding and determines different signaling profiles of human and bovine TSH. *Endocrinology* (2011) 152:3986–96. doi:10.1210/en.2011-1389
199. Mizutori Y, Chen CR, McLachlan SM, Rapoport B. The thyrotropin receptor hinge region is not simply a scaffold for the leucine-rich domain but contributes to ligand binding and signal transduction. *Mol Endocrinol* (2008) 22:1171–82. doi:10.1210/me.2007-0407
200. Mueller S, Kleinau G, Jaeschke H, Neumann S, Krause G, Paschke R. Significance of ectodomain cysteine boxes 2 and 3 for the activation mechanism of the thyroid-stimulating hormone receptor. *J Biol Chem* (2006) 281:31638–46. doi:10.1074/jbc.M604770200
201. Vlaeminck-Guillem V, Ho SC, Rodien P, Vassart G, Costagliola S. Activation of the cAMP pathway by the TSH receptor involves switching of the ectodomain from a tethered inverse agonist to an agonist. *Mol Endocrinol* (2002) 16:736–46. doi:10.1210/mend.16.4.0816
202. Schaarschmidt J, Huth S, Meier R, Paschke R, Jaeschke H. Influence of the hinge region and its adjacent domains on binding and signaling patterns of the thyrotropin and follitropin receptor. *PLoS One* (2014) 9:e111570. doi:10.1371/journal.pone.0111570
203. Chen CR, Chazenbalk GD, McLachlan SM, Rapoport B. Evidence that the C terminus of the A subunit suppresses thyrotropin receptor constitutive activity. *Endocrinology* (2003) 144:3821–7. doi:10.1210/en.2003-0430
204. Zhang M, Tong KP, Fremont V, Chen J, Narayan P, Puett D, et al. The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. *Endocrinology* (2000) 141:3514–7. doi:10.1210/endo.141.9.7790
205. Zhang ML, Sugawa H, Kosugi S, Mori T. Constitutive activation of the thyrotropin receptor by deletion of a portion of the extracellular domain. *Biochem Biophys Res Commun* (1995) 211:205–10. doi:10.1006/bbrc.1995.1797
206. Schoneberg T, Kleinau G, Bruser A. What are they waiting for? – tethered agonism in G protein-coupled receptors. *Pharmacol Res* (2016) 108:9–15. doi:10.1016/j.phrs.2016.03.027
207. Kleinau G, Jaeschke H, Mueller S, Raaka BM, Neumann S, Paschke R, et al. Evidence for cooperative signal triggering at the extracellular loops of the TSH receptor. *FASEB J* (2008) 22:2798–808. doi:10.1096/fj.07-104711
208. Ahuja S, Smith SO. Multiple switches in G protein-coupled receptor activation. *Trends Pharmacol Sci* (2009) 30:494–502. doi:10.1016/j.tips.2009.06.003
209. Kobilka B, Schertler GF. New G-protein-coupled receptor crystal structures: insights and limitations. *Trends Pharmacol Sci* (2008) 29:79–83. doi:10.1016/j.tips.2007.11.009
210. Kobilka BK. Structural insights into adrenergic receptor function and pharmacology. *Trends Pharmacol Sci* (2011) 32:213–8. doi:10.1016/j.tips.2011.02.005
211. Kobilka BK, Deupi X. Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci* (2007) 28:397–406. doi:10.1016/j.tips.2007.06.003
212. Karges B, Krause G, Homoki J, Debatin KM, de Roux N, Karges W. TSH receptor mutation V509A causes familial hyperthyroidism by release of interhelical constraints between transmembrane helices TMH3 and TMH5. *J Endocrinol* (2005) 186:377–85. doi:10.1677/joe.1.06208
213. Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, Sanborn AL, et al. Structural insights into mu-opioid receptor activation. *Nature* (2015) 524:315–21. doi:10.1038/nature14886
214. Sykiotis GP, Neumann S, Georgopoulos NA, Sgourou A, Papachatzopoulou A, Markou KB, et al. Functional significance of the thyrotropin receptor germline polymorphism D727E. *Biochem Biophys Res Commun* (2003) 301:1051–6. doi:10.1016/S0006-291X(03)00071-8

215. Bokoch MP, Zou Y, Rasmussen SG, Liu CW, Nygaard R, Rosenbaum DM, et al. Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature* (2010) 463:108–12. doi:10.1038/nature08650
216. Claus M, Jaeschke H, Kleinau G, Neumann S, Krause G, Paschke R. A hydrophobic cluster in the center of the third extracellular loop is important for thyrotropin receptor signaling. *Endocrinology* (2005) 146:5197–203. doi:10.1210/en.2005-0713
217. Neumann S, Claus M, Paschke R. Interactions between the extracellular domain and the extracellular loops as well as the 6th transmembrane domain are necessary for TSH receptor activation. *Eur J Endocrinol* (2005) 152:625–34. doi:10.1530/eje.1.01891
218. Claeysen S, Govaerts C, Lefort A, Van Sande J, Costagliola S, Pardo L, et al. A conserved Asn in TM7 of the thyrotropin receptor is a common requirement for activation by both mutations and its natural agonist. *FEBS Lett* (2002) 517:195–200. doi:10.1016/S0014-5793(02)02620-0
219. Neumann S, Krause G, Chey S, Paschke R. A free carboxylate oxygen in the side chain of position 674 in transmembrane domain 7 is necessary for TSH receptor activation. *Mol Endocrinol* (2001) 15:1294–305. doi:10.1210/mend.15.8.0672
220. Biebermann H, Schoneberg T, Schulz A, Krause G, Gruters A, Schultz G, et al. A conserved tyrosine residue (Y601) in transmembrane domain 5 of the human thyrotropin receptor serves as a molecular switch to determine G-protein coupling. *FASEB J* (1998) 12:1461–71.
221. Claus M, Neumann S, Kleinau G, Krause G, Paschke R. Structural determinants for G-protein activation and specificity in the third intracellular loop of the thyroid-stimulating hormone receptor. *J Mol Med* (2006) 84:943–54. doi:10.1007/s00109-006-0087-8
222. Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, et al. Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature* (1993) 365:649–51. doi:10.1038/365649a0
223. Angel TE, Chance MR, Palczewski K. Conserved waters mediate structural and functional activation of family A (rhodopsin-like) G protein-coupled receptors. *Proc Natl Acad Sci U S A* (2009) 106:8555–60. doi:10.1073/pnas.0903545106
224. Katritch V, Fenalti G, Abola EE, Roth BL, Cherezov V, Stevens RC. Allosteric sodium in class A GPCR signaling. *Trends Biochem Sci* (2014) 39:233–44. doi:10.1016/j.tibs.2014.03.002
225. Mason JS, Bortolato A, Congreve M, Marshall FH. New insights from structural biology into the druggability of G protein-coupled receptors. *Trends Pharmacol Sci* (2012) 33:249–60. doi:10.1016/j.tips.2012.02.005
226. Salon JA, Lodowski DT, Palczewski K. The significance of G protein-coupled receptor crystallography for drug discovery. *Pharmacol Rev* (2011) 63:901–37. doi:10.1124/pr.110.003350
227. Yuan S, Palczewski K, Peng Q, Kolinski M, Vogel H, Filipek S. The mechanism of ligand-induced activation or inhibition of mu- and kappa-opioid receptors. *Angew Chem Int Ed Engl* (2015) 54:7560–3. doi:10.1002/anie.201501742
228. Blankenship E, Vahedi-Faridi A, Lodowski DT. The high-resolution structure of activated opsin reveals a conserved solvent network in the transmembrane region essential for activation. *Structure* (2015) 23:2358–64. doi:10.1016/j.str.2015.09.015
229. Standfuss J, Edwards PC, D'Antona A, Fransen M, Xie G, Oprian DD, et al. The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature* (2011) 471:656–60. doi:10.1038/nature09795
230. Haas AK, Kleinau G, Hoyer I, Neumann S, Furkert J, Rutz C, et al. Mutations that silence constitutive signaling activity in the allosteric ligand-binding site of the thyrotropin receptor. *Cell Mol Life Sci* (2011) 68:159–67. doi:10.1007/s00018-010-0451-2
231. Hoyer I, Haas AK, Kreuchwig A, Schulein R, Krause G. Molecular sampling of the allosteric binding pocket of the TSH receptor provides discriminative pharmacophores for antagonist and agonists. *Biochem Soc Trans* (2013) 41:213–7. doi:10.1042/BST20120319
232. Chazenbalk GD, Nagayama Y, Russo D, Wadsworth HL, Rapoport B. Functional analysis of the cytoplasmic domains of the human thyrotropin receptor by site-directed mutagenesis. *J Biol Chem* (1990) 265:20970–5.
233. Kosugi S, Kohn LD, Akamizu T, Mori T. The middle portion in the second cytoplasmic loop of the thyrotropin receptor plays a crucial role in adenylate cyclase activation. *Mol Endocrinol* (1994) 8:498–509. doi:10.1210/me.8.4.498
234. Kosugi S, Mori T. The amino-terminal half of the cytoplasmic tail of the thyrotropin receptor is essential for full activities of receptor function. *Biochem Biophys Res Commun* (1994) 200:401–7. doi:10.1006/bbrc.1994.1463
235. Kosugi S, Mori T. The first cytoplasmic loop of the thyrotropin receptor is important for phosphoinositide signaling but not for agonist-induced adenylate cyclase activation. *FEBS Lett* (1994) 341:162–6. doi:10.1016/0014-5793(94)80449-4
236. Camilot M, Teofoli F, Gandini A, Franceschi R, Rapa A, Corrias A, et al. Thyrotropin receptor gene mutations and TSH resistance: variable expressivity in the heterozygotes. *Clin Endocrinol (Oxf)* (2005) 63:146–51. doi:10.1111/j.1365-2265.2005.02314.x
237. Cangul H, Morgan NV, Forman JR, Saglam H, Aycan Z, Yakut T, et al. Novel TSHR mutations in consanguineous families with congenital nongoitrous hypothyroidism. *Clin Endocrinol (Oxf)* (2010) 73:671–7. doi:10.1111/j.1365-2265.2010.03849.x
238. de Roux N, Misrahi M, Brauner R, Houang M, Carel JC, Granier M, et al. Four families with loss of function mutations of the thyrotropin receptor. *J Clin Endocrinol Metab* (1996) 81:4229–35. doi:10.1210/jcem.81.12.8954020
239. Nishihara E, Nagayama Y, Amino N, Hishinuma A, Takano T, Yoshida H, et al. A novel thyrotropin receptor germline mutation (Asp617Tyr) causing hereditary hyperthyroidism. *Endocr J* (2007) 54:927–34. doi:10.1507/endocrj.K07-088
240. Venkatakrishnan AJ, Deupi X, Lebon G, Heydenreich FM, Flock T, Miljus T, et al. Diverse activation pathways in class A GPCRs converge near the G-protein-coupling region. *Nature* (2016) 536:484–7. doi:10.1038/nature19107
241. Oldham WM, Van Eps N, Preininger AM, Hubbell WL, Hamm HE. Mechanism of the receptor-catalyzed activation of heterotrimeric G proteins. *Nat Struct Mol Biol* (2006) 13:772–7. doi:10.1038/nsmb1129
242. Smrcka AV. G protein betagamma subunits: central mediators of G protein-coupled receptor signaling. *Cell Mol Life Sci* (2008) 65:2191–214. doi:10.1007/s00018-008-8006-5
243. Neumann S, Krause G, Claus M, Paschke R. Structural determinants for g protein activation and selectivity in the second intracellular loop of the thyrotropin receptor. *Endocrinology* (2005) 146:477–85. doi:10.1210/en.2004-1045
244. Chang WC, Liao CY, Chen WC, Fan YC, Chiu SJ, Kuo HC, et al. R450H TSH receptor mutation in congenital hypothyroidism in Taiwanese children. *Clin Chim Acta* (2012) 413:1004–7. doi:10.1016/j.cca.2012.02.027
245. Mizuno H, Kanda K, Sugiyama Y, Imamine H, Ito T, Kato I, et al. Longitudinal evaluation of patients with a homozygous R450H mutation of the TSH receptor gene. *Horm Res* (2009) 71:318–23. doi:10.1159/000223415
246. Nagashima T, Murakami M, Onigata K, Morimura T, Nagashima K, Mori M, et al. Novel inactivating missense mutations in the thyrotropin receptor gene in Japanese children with resistance to thyrotropin. *Thyroid* (2001) 11:551–9. doi:10.1089/105072501750302859
247. Narumi S, Nagasaki K, Ishii T, Muroya K, Asakura Y, Adachi M, et al. Non-classic TSH resistance: TSHR mutation carriers with discrepantly high thyroidal iodine uptake. *J Clin Endocrinol Metab* (2011) 96:E1340–5. doi:10.1210/jc.2011-0070
248. Sounier R, Mas C, Steyaert J, Laeremans T, Manglik A, Huang W, et al. Propagation of conformational changes during mu-opioid receptor activation. *Nature* (2015) 524:375–8. doi:10.1038/nature14680
249. Van Sande J, Parma J, Tonacchera M, Swillens S, Dumont J, Vassart G. Somatic and germline mutations of the TSH receptor gene in thyroid diseases. *J Clin Endocrinol Metab* (1995) 80:2577–85. doi:10.1210/jc.80.9.2577
250. Harikumar KG, Morfis MM, Sexton PM, Miller LJ. Pattern of intra-family hetero-oligomerization involving the G-protein-coupled secretin receptor. *J Mol Neurosci* (2008) 36:279–85. doi:10.1007/s12031-008-9060-z
251. Li X, Staszewski L, Xu H, Durick K, Zoller M, Adler E. Human receptors for sweet and umami taste. *Proc Natl Acad Sci U S A* (2002) 99:4692–6. doi:10.1073/pnas.072090199
252. Ng HK, Chow BK. Oligomerization of family B GPCRs: exploration in inter-family oligomer formation. *Front Endocrinol* (2015) 6:10. doi:10.3389/fendo.2015.00010
253. Ng SY, Lee LT, Chow BK. Receptor oligomerization: from early evidence to current understanding in class B GPCRs. *Front Endocrinol* (2012) 3:175. doi:10.3389/fendo.2012.00175

254. Ciruela F, Vilardaga JP, Fernandez-Duenas V. Lighting up multiprotein complexes: lessons from GPCR oligomerization. *Trends Biotechnol* (2010) 28:407–15. doi:10.1016/j.tibtech.2010.05.002
255. Smith NJ, Milligan G. Allostery at G protein-coupled receptor homo- and heteromers: uncharted pharmacological landscapes. *Pharmacol Rev* (2010) 62:701–25. doi:10.1124/pr.110.002667
256. White JF, Grodnitzky J, Louis JM, Trinh LB, Shiloach J, Gutierrez J, et al. Dimerization of the class A G protein-coupled neurotensin receptor NTS1 alters G protein interaction. *Proc Natl Acad Sci U S A* (2007) 104:12199–204. doi:10.1073/pnas.0705312104
257. Kleinau G, Muller A, Biebermann H. Oligomerization of GPCRs involved in endocrine regulation. *J Mol Endocrinol* (2016) 57:R59–80. doi:10.1530/JME-16-0049
258. Ploier B, Caro LN, Morizumi T, Pandey K, Pearring JN, Goren MA, et al. Dimerization deficiency of enigmatic retinitis pigmentosa-linked rhodopsin mutants. *Nat Commun* (2016) 7:12832. doi:10.1038/ncomms12832
259. Rivero-Muller A, Chou YY, Ji I, Lajic S, Hanyaloglu AC, Jonas K, et al. Rescue of defective G protein-coupled receptor function in vivo by intermolecular cooperation. *Proc Natl Acad Sci U S A* (2010) 107:2319–24. doi:10.1073/pnas.0906695106
260. Tadagaki K, Jockers R, Kamal M. History and biological significance of GPCR heteromerization in the neuroendocrine system. *Neuroendocrinology* (2012) 95:223–31. doi:10.1159/000330000
261. Tadagaki K, Tudor D, Gbahou F, Tschische P, Waldhoer M, Bomsel M, et al. Human cytomegalovirus-encoded UL33 and UL78 heteromerize with host CCR5 and CXCR4 impairing their HIV coreceptor activity. *Blood* (2012) 119:4908–18. doi:10.1182/blood-2011-08-372516
262. Tschische P, Tadagaki K, Kamal M, Jockers R, Waldhoer M. Heteromerization of human cytomegalovirus encoded chemokine receptors. *Biochem Pharmacol* (2011) 82:610–9. doi:10.1016/j.bcp.2011.06.009
263. Levoye A, Dam J, Ayoub MA, Guillaume JL, Couturier C, Delagrange P, et al. The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO J* (2006) 25:3012–23. doi:10.1038/sj.emboj.7601193
264. Lohse MJ. Dimerization in GPCR mobility and signaling. *Curr Opin Pharmacol* (2010) 10:53–8. doi:10.1016/j.coph.2009.10.007
265. Bouvier M. Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* (2001) 2:274–86. doi:10.1038/35067575
266. George SR, O'Dowd BF, Lee SP. G-protein-coupled receptor oligomerization and its potential for drug discovery. *Nat Rev Drug Discov* (2002) 1:808–20. doi:10.1038/nrd913
267. Uberti MA, Hague C, Oller H, Minneman KP, Hall RA. Heterodimerization with beta2-adrenergic receptors promotes surface expression and functional activity of alpha1D-adrenergic receptors. *J Pharmacol Exp Ther* (2005) 313:16–23. doi:10.1124/jpet.104.079541
268. Persani L, Calebiro D, Bonomi M. Technology Insight: modern methods to monitor protein-protein interactions reveal functional TSH receptor oligomerization. *Nat Clin Pract Endocrinol Metab* (2007) 3:180–90. doi:10.1038/ncpendmet0401
269. Allen MD, Neumann S, Gershengorn MC. Occupancy of both sites on the thyrotropin (TSH) receptor dimer is necessary for phosphoinositide signaling. *FASEB J* (2011) 25:3687–94. doi:10.1096/fj.11-188961
270. Latif R, Graves P, Davies TF. Ligand-dependent inhibition of oligomerization at the human thyrotropin receptor. *J Biol Chem* (2002) 277:45059–67. doi:10.1074/jbc.M206693200
271. Davies T, Marians R, Latif R. The TSH receptor reveals itself. *J Clin Invest* (2002) 110:161–4. doi:10.1172/JCI0216234
272. Tenenbaum-Rakover Y, Grasberger H, Mamanasiri S, Ringkananont U, Montanelli L, Barkoff MS, et al. Loss-of-function mutations in the thyrotropin receptor gene as a major determinant of hyperthyrotropinemia in a consanguineous community. *J Clin Endocrinol Metab* (2009) 94:1706–12. doi:10.1210/jc.2008-1938
273. Calebiro D, de Filippis T, Lucchi S, Covino C, Panigone S, Beck-Peccoz P, et al. Intracellular entrapment of wild-type TSH receptor by oligomerization with mutants linked to dominant TSH resistance. *Hum Mol Genet* (2005) 14:2991–3002. doi:10.1093/hmg/ddi329
274. Biebermann H, Winkler F, Handke D, Teichmann A, Gerling B, Cameron F, et al. New pathogenic thyrotropin receptor mutations decipher differentiated activity switching at a conserved helix 6 motif of family A GPCR. *J Clin Endocrinol Metab* (2012) 97:E228–32. doi:10.1210/jc.2011-2106
275. Guo W, Shi L, Filizola M, Weinstein H, Javitch JA. Crosstalk in G protein-coupled receptors: changes at the transmembrane homodimer interface determine activation. *Proc Natl Acad Sci U S A* (2005) 102:17495–500. doi:10.1073/pnas.0508950102
276. Bakker RA, Dees G, Carrillo JJ, Booth RG, Lopez-Gimenez JF, Milligan G, et al. Domain swapping in the human histamine H1 receptor. *J Pharmacol Exp Ther* (2004) 311:131–8. doi:10.1124/jpet.104.067041
277. Mancia F, Assur Z, Herman AG, Siegel R, Hendrickson WA. Ligand sensitivity in dimeric associations of the serotonin 5HT2c receptor. *EMBO Rep* (2008) 9:363–9. doi:10.1038/embor.2008.27
278. Gorinski N, Kowalsman N, Renner U, Wirth A, Reinartz MT, Seifert R, et al. Computational and experimental analysis of the transmembrane domain 4/5 dimerization interface of the serotonin 5-HT(1A) receptor. *Mol Pharmacol* (2012) 82:448–63. doi:10.1124/mol.112.079137
279. George SR, Lee SP, Varghese G, Zeman PR, Seeman P, Ng GY, et al. A transmembrane domain-derived peptide inhibits D1 dopamine receptor function without affecting receptor oligomerization. *J Biol Chem* (1998) 273:30244–8. doi:10.1074/jbc.273.46.30244
280. Hu J, Hu K, Liu T, Stern MK, Mistry R, Challiss RA, et al. Novel structural and functional insights into M3 muscarinic receptor dimer/oligomer formation. *J Biol Chem* (2013) 288:34777–90. doi:10.1074/jbc.M113.503714
281. Yanagawa M, Yamashita T, Shichida Y. Comparative fluorescence resonance energy transfer analysis of metabotropic glutamate receptors: implications about the dimeric arrangement and rearrangement upon ligand bindings. *J Biol Chem* (2011) 286:22971–81. doi:10.1074/jbc.M110.206870
282. Latif R, Michalek K, Morshed SA, Davies TF. A tyrosine residue on the TSH receptor stabilizes multimer formation. *PLoS One* (2010) 5:e9449. doi:10.1371/journal.pone.0009449
283. Latif R, Ali MR, Mezei M, Davies TF. Transmembrane domains of attraction on the TSH receptor. *Endocrinology* (2015) 156:488–98. doi:10.1210/en.2014-1509
284. Chazenbalk GD, Kakinuma A, Jaume JC, McLachlan SM, Rapoport B. Evidence for negative cooperativity among human thyrotropin receptors overexpressed in mammalian cells. *Endocrinology* (1996) 137:4586–91. doi:10.1210/endo.137.11.8895321
285. Jiang X, Fischer D, Chen X, McKenna SD, Liu H, Sriraman V, et al. Evidence for follicle-stimulating hormone receptor as a functional trimer. *J Biol Chem* (2014) 289:14273–82. doi:10.1074/jbc.M114.549592
286. Boutin A, Allen MD, Geras-Raaka E, Huang W, Neumann S, Gershengorn MC. Thyrotropin receptor stimulates internalization-independent persistent phosphoinositide signaling. *Mol Pharmacol* (2011) 80:240–6. doi:10.1124/mol.111.072157
287. Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, Tacchetti C, et al. Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol* (2009) 7:e1000172. doi:10.1371/journal.pbio.1000172
288. Calebiro D, Nikolaev VO, Lohse MJ. Imaging of persistent cAMP signaling by internalized G protein-coupled receptors. *J Mol Endocrinol* (2010) 45:1–8. doi:10.1677/JME-10-0014
289. Calebiro D, Nikolaev VO, Persani L, Lohse MJ. Signaling by internalized G-protein-coupled receptors. *Trends Pharmacol Sci* (2010) 31:221–8. doi:10.1016/j.tips.2010.02.002
290. Calebiro D, Rieken F, Wagner J, Sungkaworn T, Zabel U, Borzi A, et al. Single-molecule analysis of fluorescently labeled G-protein-coupled receptors reveals complexes with distinct dynamics and organization. *Proc Natl Acad Sci U S A* (2012) 110(2):743–8. doi:10.1073/pnas.1205798110
291. Cianfarani F, Baldini E, Cavalli A, Marchioni E, Lembo L, Teson M, et al. TSH receptor and thyroid-specific gene expression in human skin. *J Invest Dermatol* (2010) 130:93–101. doi:10.1038/jid.2009.180
292. Davies TF, Teng CS, McLachlan SM, Smith BR, Hall R. Thyrotropin receptors in adipose tissue, retro-orbital tissue and lymphocytes. *Mol Cell Endocrinol* (1978) 9:303–10. doi:10.1016/0303-7207(78)90072-2
293. de Lloyd A, Bursell J, Gregory JW, Rees DA, Ludgate M. TSH receptor activation and body composition. *J Endocrinol* (2010) 204:13–20. doi:10.1677/JOE-09-0262
294. Liu Z, Ji Z, Wang G, Chao T, Hou L, Wang J. Genome-wide analysis reveals signatures of selection for important traits in domestic sheep from different ecoregions. *BMC Genomics* (2016) 17:863. doi:10.1186/s12864-016-3212-2

295. Martinez Barrio A, Lamichhaney S, Fan G, Rafati N, Pettersson M, Zhang H, et al. The genetic basis for ecological adaptation of the Atlantic herring revealed by genome sequencing. *Elife* (2016) 5:e12081. doi:10.7554/eLife.12081
296. Ono H, Hoshino Y, Yasuo S, Watanabe M, Nakane Y, Murai A, et al. Involvement of thyrotropin in photoperiodic signal transduction in mice. *Proc Natl Acad Sci U S A* (2008) 105:18238–42. doi:10.1073/pnas.0808952105

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer, MM, and handling editor declared their shared affiliation, and the handling editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Endocrinology

MINI REVIEW
published: 03 April 2017
doi: 10.3389/fendo.2017.00057

Genetics of Thyroid-Stimulating Hormone Receptor—Relevance for Autoimmune Thyroid Disease

Mihaela Stefan and Larissa C. Faustino*

Division of Endocrinology, Department of Medicine, Albert Einstein College of Medicine, Bronx, NY, USA

Production of thyroid-stimulating hormone receptor (TSHR) antibodies represents the hallmark of Graves' disease (GD) pathogenesis. Thus, for more than two decades the TSHR gene has been at the center of studies intended to elucidate its contribution to disease pathology. The advent of genome-wide association technology allowed to establish a strong association of the TSHR gene with GD. Subsequent fine-mapping studies narrowed the disease-susceptibility region to a 40 kb sequence in intron 1, where at least five GD-associated SNPs in tight linkage disequilibrium were identified. The current challenge is to understand the functional mechanisms by which these polymorphisms modify physiological processes and trigger disease. The aim of this review is to summarize the current knowledge on the role of the TSHR gene in GD pathogenesis, which has been gained through linkage and association studies, as well as to discuss the emerging mechanisms underlying biological implications of TSHR variants in the development of GD.

Keywords: Graves' disease, thyroid-stimulating hormone receptor, single nucleotide polymorphisms, GWAS, histone modifications

OPEN ACCESS

Edited by:
Rauf Latif,
Icahn School of Medicine at Mount Sinai, USA

Reviewed by:
Giuseppe Barbesino,
Partners HealthCare, USA
Takao Ando,
Nagasaki University Hospital, Japan

****Correspondence:***
Mihaela Stefan
mihaela.stefan@einstein.yu.edu

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *25 January 2017*
Accepted: *10 March 2017*
Published: *03 April 2017*

Citation:
Stefan M and Faustino LC (2017) Genetics of Thyroid-Stimulating Hormone Receptor—Relevance for Autoimmune Thyroid Disease. Front. Endocrinol. 8:57. doi: 10.3389/fendo.2017.00057

INTRODUCTION

Autoimmune thyroid diseases (AITD), including Graves' disease (GD) and Hashimoto's thyroiditis (HT), affect 2–5% of the general population, representing the most frequent autoimmune conditions (1, 2). Similar to other complex autoimmune diseases, it is believed that AITD occur when interactions of genetic susceptibility factors with environmental triggers lead to loss of immune self-tolerance at peripheral and central levels (3). During the last three decades, several approaches from linkage and association studies to candidate genes analysis and whole-genome screening enabled significant progress in the identification of genes that confer susceptibility to AITD. These genes are broadly grouped as either immune-modulating genes (including the HLA family, CD40, CD25, FOXP3, PTPN22) or thyroid-specific genes [thyroid-stimulating hormone receptor (TSHR) and TG].

The TSHR is unique among these susceptibility genes because it encodes for a protein that is both responsible for the clinical manifestations of the disease and is the direct target of autoimmune response in GD. The TSHR gene, located on chromosome 14q31, consists of 10 exons and encodes for a G protein-coupled receptor that plays a central role in the regulation of thyroid development, growth, and function. Indeed, TSHR-stimulating antibodies (TSAbs) are present in nearly all cases of GD, and severity of the disease correlates with TSAbs levels (4). Moreover, compelling evidence has correlated TSHR gene variants exclusively with GD susceptibility and not with HT development (5, 6). The aim of this review is to summarize the current knowledge of the role of TSHR gene in GD pathology, gained through linkage analysis, association, and functional studies.

LINKAGE STUDIES

Whole-genome linkage is a powerful technique for screening the human genome for major susceptibility genes, without any previous assumption on the mechanisms of genetic susceptibility to the disease. It is based on the principle that the probability of recombination between two loci is directly related to the genetic distance between them. Thus, if two loci comprising a polymorphism and a disease-related gene are close to each other on a chromosome, the alleles of the polymorphic loci will cosegregate with the disease within affected families because the likelihood of recombination between the polymorphism and the disease-related gene is very low. Single nucleotide polymorphisms (SNPs) and microsatellites are the main genetic polymorphisms screened in the linkage studies (7, 8).

Several linkage studies intended to detect GD-specific loci had limited success in demonstrating significant linkage (as well as association) of the TSHR gene with GD (9–13). However, Tomer et al. identified a large region of linkage on chromosome 14q31 containing the TSHR locus in whole-genome linkage studies in multiplex, multigenerational AITD families (14–17). They identified a locus, designated Graves' disease 1 (GD-1) that included TSHR gene, as well as other potential candidate genes—such as estrogen receptor 2, deiodinase type 2, and immunoglobulin heavy locus (14, 17). Later, fine-mapping analysis by Tomer et al. confirmed that the susceptibility gene in the GD-1 locus was indeed the TSHR gene, even though a second gene in this locus, NRXN3, was also identified as a major GD gene (18).

ASSOCIATION STUDIES

Reflecting the importance of TSHR for GD pathogenesis, TSHR was the first non-MHC gene to be tested for association with the disease. Three germline missense mutations were initially described in patients with GD and proposed to be associated with the disease (19, 20): a substitution of aspartic acid (D) for histidine (H) in position 36 (D36H); a substitution of a proline (P) for threonine (T) in position 52 (P52T), and a substitution of aspartic acid (D) for glutamic acid (E) in position 727 (D727E). Two of these three mutations, D36H and P52T, are located in the putative ligand binding region of the extracellular domain of the TSHR, while the third one, D727E lies within the intracellular domain of the receptor.

However, studies regarding association of these SNPs with GD were contradictory. In 1995, Bahn and colleagues were first to report the association of the P52T polymorphism with AITD in female population (21), but in a subsequent study Watson et al. found no differences in the distribution of this polymorphism in GD patients as compared with autoimmune hypothyroidism patients and control individuals (22). Several subsequent studies reached inconsistent results, thus a clear association of P52T or D36H with GD was not confirmed (23–25). In 1999, Gabriel et al. reported that the C to G transition in the TSHR 727 codon leading to D727E variant has an increased frequency in patients with non-autoimmune thyroiditis (26), and this association was also recently reported in a small Turkish population (27). However, the D727E association with non-autoimmune thyroiditis was not supported by studies in large series of European Caucasian patients (28). The association of D727E polymorphism with autoimmune thyroiditis was supported by data from a case–control study in Russian populations (29) but was not confirmed in US Caucasian patients (13, 26). Finally, Tomer group performed a case–control study and meta-analysis combining the data from three independent studies and showed a very week association of D727E polymorphism and GD (13).

To date, no compelling evidence exists to support a role of these three TSHR polymorphisms in GD pathogenesis. Given the frequency of these variants in general population, it is believed that they are common polymorphisms, not implicated in development of GD (20). The lack of consistency among completed studies could be the consequence of ethnic differences, selection bias, and population stratification.

In the last decade, the association of common genetic variants with complex diseases was significantly facilitated by the increased ability to measure genetic variability of hundreds of markers in large cohorts of individuals. The advent of genome-wide association technology applied to large case–control studies allowed identification of disease-associated variants and their contribution to disease susceptibility. Applying this technology to AITD resulted in identification of new disease-associated loci, including TSHR, and provided unique insights into their genetic contribution to disease pathology. **Table 1** summarizes the main studies conducted over the years that established association of the TSHR gene with GD risk.

In 2005, Dechairo et al. analyzed 40 SNPs mapping to a 600-kb sequence encompassing the TSHR gene in a Caucasian cohort of 1,056 AITD patients and 971 controls. They identified a haplotype associated with GD (OR = 1.7), but not with autoimmune hypothyroidism, and concluded that TSHR is a GD-specific susceptibility locus (5). Importantly, rs2268458, the SNP showing the strongest association (OR = 1.3) with GD mapped to TSHR intron 1; the association was confirmed in a large UK Caucasian cohort (5). The same year, an independent case–control study conducted in 400 patients with AITD and 238 controls of Japanese descent found several adjacent SNPs in TSHR intron 7 significantly associated with GD, but not with autoimmune hypothyroidism, suggesting that polymorphisms in the TSHR intron 7 could contribute to GD susceptibility (32). However, subsequent association studies conducted over several years in Caucasian populations could not replicate the association of TSHR intron 7 SNPs with GD (6, 35, 41).

In 2009, Brand et al. interrogated a panel of 98 SNPs spanning an 800 kb region of the TSHR gene in a cohort of 768 GD subjects and 768 controls (6). The SNPs showing the strongest association with GD, rs179247 (OR = 1.53), and rs12101255 (OR = 1.55) were located in TSHR intron 1 (6). The association of the TSHR intron 1 SNPs with GD was validated by further studies of several Caucasian (34, 35) and Brazilian (41) populations. In 2011, a large GWAS conducted by the China Consortium of the Genetics of Autoimmune Thyroid Disease in 1,536 individuals with GD and 1,516 controls confirmed TSHR as a primary susceptibility locus for GD by finding a robust association (OR = 1.35) of an intron 1 SNP (rs12101261) with the disease (36). Two years later, the

TABLE 1 | Association studies of thyroid-stimulating hormone receptor (TSHR) gene with Graves' disease risk.

Studies	Cases (*n*)	Population	Main polymorphisms found	Associated TSHR region
Cuddihy et al. (30)	91	Caucasian (USA)	rs2234919 (P52T)	Exon 1
Akamizu et al. (31)	186	Japanese	TSHR-AT	Intron 2
Chistiakov et al. (29)	78	Russian	rs1991517 (D727E)	Exon 10
Ho et al. (24)	164	Chinese, Malays, Indians	rs2239610	Intron 1
Hiratani et al. (32)	250	Japanese	rs2268475, rs3783938	Intron 7, intron 8
Dechairo et al. (5)	1,422	Caucasian (UK)	rs2268458	Intron 1
Burton et al. (33)	1,000	Caucasian (UK)	rs3783941	Intron 8
Yin et al. (34)	200	Caucasian (women only)	rs2268458	Intron 1
Brand et al. (6)	768	Caucasian (UK)	rs179247, rs12101255	Intron 1
Ploski et al. (35)	3,258	Caucasian (Poland, UK)	rs179247, rs12101255	Intron 1
Chu et al. (36)	5,530	Chinese	rs12101261	Intron 1
Colobran et al. (37)	137	Caucasian (Spanish)	rs179247	Intron 1
Liu et al. (38)	404	Chinese	rs12101255, rs179247	Intron 1
Inoue et al. (39)	112	Japanese	rs179247	Intron 1
Tomer et al. (18)	225	Caucasian (USA)	rs2284720	Intron 1
Liu et al. (40)	5,368	Chinese	rs12101261, rs179243	Intron 1
Bufalo et al. (41)	279	Brazilian	rs179247, rs12885526	Intron 1
Fujii et al. (42)	180	Japanese	rs4411444	Intron 1
Lombardi et al. (43)	333	Caucasian (Italy)	rs179247, rs3783948, rs12101255	Intron 1

same group conducted a fine-mapping study that established the association of two independent TSHR intron 1 susceptibility variants, rs1201261 and rs179243 in a large Chinese Han population (40). Noticeable, rs1201261 and rs179243 are in tight linkage disequilibrium (LD) with rs12101255 ($r^2 = 1.0$) and rs2268458 ($r^2 = 0.91$), respectively, which were found associated with GD in Caucasian populations of European descent (6).

Recently, three meta-analysis studies intended to refine the effects of rs179247 and rs12101255 SNPs on GD susceptibility concluded that there is a significant association between these SNPs in TSHR intron 1 and GD (44–46).

Collectively, the association studies conducted during the last decade provided compelling evidence and established TSHR as a GD-specific susceptibility locus. Furthermore, fine-mapping studies pointed to a unique susceptibility region located in TSHR intron 1, where at least five GD-associated SNPs were mapped: rs179247, rs2284720, rs12101255, rs12101261, and rs2268458 (**Figure 1A**).

FUNCTIONAL RELEVANCE OF TSHR POLYMORPHISMS

The discovery of the TSHR GD-associated SNPs within a non-protein coding gene region raised questions about their potential effect on gene function, as well as their impact on disease pathology. Since DNA variants located in intronic or intergenic sites can impact different layers of gene regulation, identification of their functional role is often difficult. For example, by modifying the DNA sequence, non-coding SNPs can affect transcriptional factors' (TFs) binding and thus modulate gene transcription; they can also impact RNA splicing and stability as well as posttranslation events (48). In addition, DNA variants can modulate, directly or indirectly, epigenetic marks such as DNA methylation, histone modifications, and microRNAs activity (48, 49). Recently, it has been shown that differential binding of TFs at sites harboring DNA variants triggers specific histone modifications, which can modify gene expression and determine the phenotype (50–52). In the case of disease-associated SNPs, such genetic–epigenetic interactions can increase the risk or even trigger disease in certain individuals.

To date, studies aimed to unveil the mechanistic role of TSHR intron 1 variants in gene function and thyroid autoimmunity pointed to two distinct mechanisms. The first proposed mechanism supports a role of the disease-associated SNPs in defective peripheral tolerance; the second mechanism supports the concept that the disease-associated intron 1 SNPs cause reduced intrathymic TSHR expression, leading to decreased central tolerance and increased risk of autoimmunity to TSHR.

Supportive of the first mechanism, Brand et al. proposed that the TSHR intron 1 GD-associated SNPs regulate mRNA spicing, resulting in increased levels of variants encoding a more autoantigenic TSHR A-subunit (6). The authors measured the levels of full length TSHR (flTSHR) mRNA and of two TSHR truncated transcripts named ST4 and ST5 in thyroid tissues of 12 individuals and showed that the disease-risk alleles of 2 intron 1 SNPs (rs179247 and rs12101255) associate with increased ST4 and ST5 and with decreased flTSHR levels. They suggested that the truncated ST4 and ST5 variants could be translated into TSHR extracellular A-subunit, the main target of TSHR autoantibodies (6). However, the mechanisms by which the two SNPs interact with TSHR mRNA splicing were not addressed, and the authors did not exclude the possibility that other intron 1 SNPs in strong LD could also modulate TSHR transcription (53).

The second mechanism by which TSHR intron 1 variants could trigger thyroid autoimmunity through defective central tolerance was initially proposed by Pujol-Borrell group (37). By measuring TSHR mRNA levels in thymus and correlating them with the genotype of intron 1 SNPs, Colobran et al. found that individuals carrying the disease-protective genotype at the rs179247 site have higher levels of thymic TSHR mRNA than those with the disease-associated genotype (37). These findings, coupled with the fact that negative selection of autoreactive thymocytes is dose dependent (54), support the concept that, by modulating TSHR transcription, intron 1 disease-associated SNPs could modulate negative selection of TSHR-autoreactive T cells in the thymus. Thus, decreased TSHR thymic expression

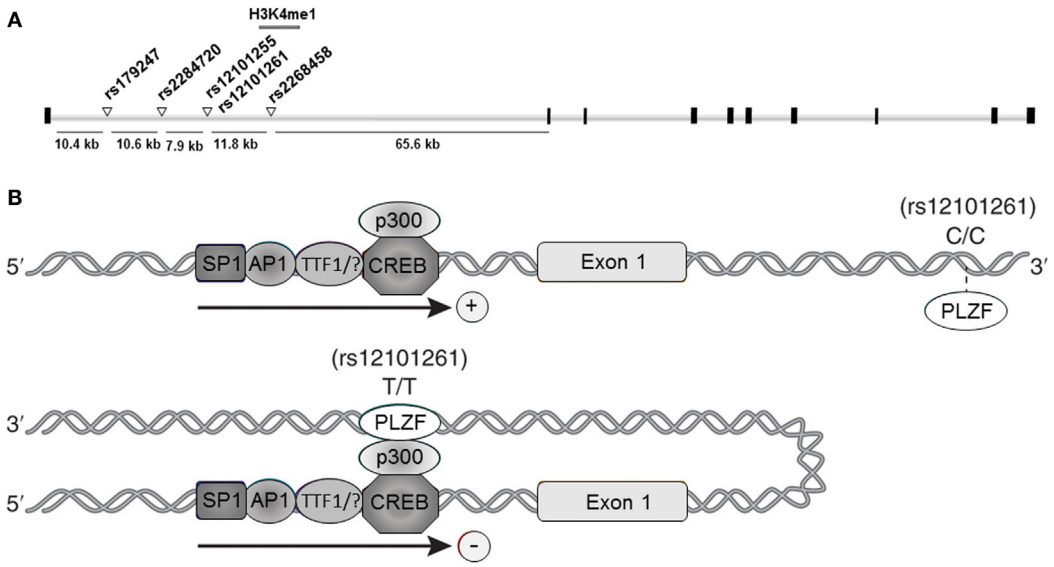


FIGURE 1 | **Thyroid-stimulating hormone receptor (TSHR) intron 1 Graves' disease (GD)-associated DNA variants**. **(A)** Schematic representation of the TSHR gene and the intron 1 GD-associated single nucleotide polymorphisms (SNPs). Black squares represent exons; white bars represent introns; white triangles in intron 1 represent the SNPs associated with GD susceptibility; gray bar overlapping the rs12101255 and rs12101261 represent the region characterized by H3K4me1 enrichment (47). **(B)** Proposed model for rs12101261 allele-dependent regulation of TSHR transcription. Upper panel: the presence of the disease-protective genotype (C/C) at the rs12101261 site prevents strong interactions with promyelocytic leukemia zinc finger protein (PLZF), allowing TSHR transcription; lower panel: the presence of the disease-associated genotype (T/T) facilitates binding of PLZF, triggering chromatin folding and interaction with TSHR promoter and transcriptional machinery.

would facilitate the escape of TSHR-reactive T cells from central tolerance in genetically susceptible individuals, increasing the risk for AITD development. The same group showed that TSHR mRNA and protein are expressed in thymocytes from early stages of differentiation but are not detected in extra-thymic T cells. Moreover, thymic TSHR is functional, and TSAbs from GD patients can stimulate thymocytes through this receptor (55). Based on these findings, Pujol-Borrell group suggested that constant stimulation of thymic TSHR by TSAbs can be a potential mechanism explaining thymic hyperplasia, commonly observed in GD (55). Furthermore, the authors proposed that continuous TSAbs stimulation of thymocytes could lead to improved affinity and stimulating capability of TSHR cross-reactive low-affinity antibodies due to the interactions between egressing thymocytes and B-cells in the lymph nodes or the thyroid gland (55, 56). This would result in production of high-affinity TSAbs, the hallmark of GD pathogenesis.

Mechanistic insights into the contribution of TSHR intron 1 SNPs to AITD susceptibility through defective central tolerance were recently revealed by studies from Tomer group (47). Their work originated from the premise that the disease-associated variants can specifically interact, through epigenetic modifications, with environmental factors to trigger disease susceptibility. To reveal the functionality of the GD-associated SNPs, Stefan et al. (47) analyzed genome-wide modifications of histone 3 lysine 4 (K4)-monomethylated (H3K4me1), a chromatin mark often associated with distal enhancer elements (57), induced by interferon alpha (IFNα), a key cytokine secreted during viral infections, previously shown to trigger thyroid autoimmunity (58).

This approach led to identification of an open chromatin region marked by IFNα-induced H3K4me1 enrichment overlapping two adjacent TSHR intron 1 GD-associated SNPs: rs12101255 and rs12101261 (47) (**Figure 1A**). Functional studies revealed that the region overlapping the rs12101261 site harbors a regulatory element that functions through binding of the transcriptional repressor, promyelocytic leukemia zinc finger protein (PLZF). PLZF binding was shown to be restricted at the disease-associated variant of the rs12101261 site and was correlated with lower thymic TSHR mRNA levels in individuals carrying the disease-predisposing genotype, as compared with individuals carrying the disease-protective genotype. The authors proposed that loss of proper genetic–epigenetic interactions due to microenvironmental influences, such as sustained IFNα production during viral infections, would affect the regulation of TSHR susceptible variants resulting in impaired gene expression. In thymus, the lower TSHR expression triggered by the susceptible genotype would likely facilitate escape from central tolerance and increases the risk of autoimmunity to TSHR.

However, the underlying mechanisms by which the *cis*-regulatory element at the rs12101261 site modulates TSHR transcription have still to be experimentally addressed. A possible regulatory model involves long-range chromatin interactions determined by the rs12101261 genotype. In such model, the presence of the disease-protective genotype (C/C) would cause a weak interaction of PLZF with chromatin at the rs12101261 site, resulting in active TSHR transcription (**Figure 1B**, upper panel). The presence of the disease-associated genotype (T/T) would enable strong PLZF binding at the *cis*-element, triggering

formation of chromatin loops and direct interactions between PLZF and TSHR promoter, resulting in inhibition of transcription (**Figure 1B**, lower panel). Thus, allele-dependent differences in chromatin folding would trigger allele-dependent differences in gene expression. These chromatin interactions, still to be experimentally addressed, are likely cell specific, and different factors (e.g., TFs) and SNPs may control TSHR expression in different tissues.

CONCLUDING REMARKS

Studies in the last 15 years established a robust association of TSHR gene with GD, and the disease-associated locus was recently fine-mapped within 40 kb region in intron 1. Moreover, it has become clear that whether TSHR SNPs interfere with gene expression in thymus leading to the escape of TSHR-reactive T cells from central tolerance or defects in peripheral tolerance are involved, these variants are unlikely to act alone, and interactions with epigenetic and environmental factors as well as combinatorial effects should be considered.

Although important advances have been made in our understanding of the role of TSHR polymorphisms in AITD, questions still persist. What are the mechanisms by which TSHR polymorphisms predispose to disease? Which are the environmental factors that unequivocally contribute to disease development and how they interact with susceptible variants? Can genetic variants be translated into markers predicting disease development? Does susceptibility of epigenetic markers to environmental triggers have a role in the functionality of the disease-associated variants? To answer these questions, more work and close collaborations of molecular biologists and clinical scientists as well as more integrated approaches are needed. It is hoped that such knowledge would open the road toward the development of new, targeted, and preventive therapies based on the individual's particular susceptibility.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

ACKNOWLEDGMENTS

The authors thank Dr. Yaron Tomer for reviewing the manuscript, helpful comments, and recommendations.

REFERENCES

1. McLachlan SM, Rapoport B. Breaking tolerance to thyroid antigens: changing concepts in thyroid autoimmunity. *Endocr Rev* (2014) 35:59–105. doi:10.1210/er.2013-1055
2. Lee HJ, Li CW, Hammerstad SS, Stefan M, Tomer Y. Immunogenetics of autoimmune thyroid diseases: a comprehensive review. *J Autoimmun* (2015) 64:82–90. doi:10.1016/j.jaut.2015.07.009
3. Tomer Y, Huber A. The etiology of autoimmune thyroid disease: a story of genes and environment. *J Autoimmun* (2009) 32:231–9. doi:10.1016/j.jaut.2009.02.007
4. Tomer Y. Mechanisms of autoimmune thyroid diseases: from genetics to epigenetics. *Annu Rev Pathol* (2014) 9:147–56. doi:10.1146/annurev-pathol-012513-104713
5. Dechairo BM, Zabaneh D, Collins J, Brand O, Dawson GJ, Green AP, et al. Association of the TSHR gene with Graves' disease: the first disease specific locus. *Eur J Hum Genet* (2005) 13:1223–30. doi:10.1038/sj.ejhg.5201485
6. Brand OJ, Barrett JC, Simmonds MJ, Newby PR, McCabe CJ, Bruce CK, et al. Association of the thyroid stimulating hormone receptor gene (TSHR) with Graves' disease. *Hum Mol Genet* (2009) 18:1704–13. doi:10.1093/hmg/ddp087
7. Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet* (1995) 11:241–7. doi:10.1038/ng1195-241
8. Allahabadia A, Gough SC. The different approaches to the genetic analysis of autoimmune thyroid disease. *J Endocrinol* (1999) 163:7–13. doi:10.1677/joe.0.1630007
9. de Roux N, Shields DC, Misrahi M, Ratanachaiyavong S, McGregor AM, Milgrom E. Analysis of the thyrotropin receptor as a candidate gene in familial Graves' disease. *J Clin Endocrinol Metab* (1996) 81:3483–6. doi:10.1210/jcem.81.10.8855789
10. Kotsa KD, Watson PF, Weetman AP. No association between a thyrotropin receptor gene polymorphism and Graves' disease in the female population. *Thyroid* (1997) 7:31–3. doi:10.1089/thy.1997.7.31
11. Allahabadia A, Heward JM, Mijovic C, Carr-Smith J, Daykin J, Cockram C, et al. Lack of association between polymorphism of the thyrotropin receptor gene and Graves' disease in United Kingdom and Hong Kong Chinese patients: case control and family-based studies. *Thyroid* (1998) 8:777–80. doi:10.1089/thy.1998.8.777
12. Sakai K, Shirasawa S, Ishikawa N, Ito K, Tamai H, Kuma K, et al. Identification of susceptibility loci for autoimmune thyroid disease to 5q31-q33 and Hashimoto's thyroiditis to 8q23-q24 by multipoint affected sib-pair linkage analysis in Japanese. *Hum Mol Genet* (2001) 10:1379–86. doi:10.1093/hmg/10.13.1379
13. Ban Y, Greenberg DA, Concepcion ES, Tomer Y. A germline single nucleotide polymorphism at the intracellular domain of the human thyrotropin receptor does not have a major effect on the development of Graves' disease. *Thyroid* (2002) 12:1079–83. doi:10.1089/105072502321085171
14. Tomer Y, Barbesino G, Keddache M, Greenberg DA, Davies TF. Mapping of a major susceptibility locus for Graves' disease (GD-1) to chromosome 14q31. *J Clin Endocrinol Metab* (1997) 82:1645–8. doi:10.1210/jc.82.5.1645
15. Tomer Y, Barbesino G, Greenberg DA, Concepcion E, Davies TF. Linkage analysis of candidate genes in autoimmune thyroid disease. III. Detailed analysis of chromosome 14 localizes Graves' disease-1 (GD-1) close to multinodular goiter-1 (MNG-1). International Consortium for the Genetics of Autoimmune Thyroid Disease. *J Clin Endocrinol Metab* (1998) 83:4321–7. doi:10.1210/jc.83.12.4321
16. Tomer Y, Barbesino G, Greenberg DA, Concepcion E, Davies TF. Mapping the major susceptibility loci for familial Graves' and Hashimoto's diseases: evidence for genetic heterogeneity and gene interactions. *J Clin Endocrinol Metab* (1999) 84:4656–64. doi:10.1210/jc.84.12.4656
17. Tomer Y, Ban Y, Concepcion E, Barbesino G, Villanueva R, Greenberg DA, et al. Common and unique susceptibility loci in Graves and Hashimoto diseases: results of whole-genome screening in a data set of 102 multiplex families. *Am J Hum Genet* (2003) 73:736–47. doi:10.1086/378588
18. Tomer Y, Hasham A, Davies TF, Stefan M, Concepcion E, Keddache M, et al. Fine mapping of loci linked to autoimmune thyroid disease identifies novel susceptibility genes. *J Clin Endocrinol Metab* (2013) 98:E144–52. doi:10.1210/jc.2012-2408
19. Jacobson EM, Tomer Y. The CD40, CTLA-4, thyroglobulin, TSH receptor, and PTPN22 gene quintet and its contribution to thyroid autoimmunity: back to the future. *J Autoimmun* (2007) 28:85–98. doi:10.1016/j.jaut.2007.02.006
20. Tonacchera M, Pinchera A. Thyrotropin receptor polymorphisms and thyroid diseases. *J Clin Endocrinol Metab* (2000) 85:2637–9. doi:10.1210/jcem.85.8.6801
21. Cuddihy RM, Bryant WP, Bahn RS. Normal function in vivo of a homozygotic polymorphism in the human thyrotropin receptor. *Thyroid* (1995) 5:255–7. doi:10.1089/thy.1995.5.255
22. Watson PF, French A, Pickerill AP, McIntosh RS, Weetman AP. Lack of association between a polymorphism in the coding region of the thyrotropin receptor gene and Graves' disease. *J Clin Endocrinol Metab* (1995) 80:1032–5. doi:10.1210/jc.80.3.1032

23. Simanainen J, Kinch A, Westermark K, Winsa B, Bengtsson M, Schuppert F, et al. Analysis of mutations in exon 1 of the human thyrotropin receptor gene: high frequency of the D36H and P52T polymorphic variants. *Thyroid* (1999) 9:7–11. doi:10.1089/thy.1999.9.7
24. Ho SC, Goh SS, Khoo DH. Association of Graves' disease with intragenic polymorphism of the thyrotropin receptor gene in a cohort of Singapore patients of multi-ethnic origins. *Thyroid* (2003) 13:523–8. doi:10.1089/105072503322238773
25. Chistyakov DA, Savost'anov KV, Turakulov RI, Petunina NA, Trukhina LV, Kudinova AV, et al. Complex association analysis of graves disease using a set of polymorphic markers. *Mol Genet Metab* (2000) 70:214–8. doi:10.1006/mgme.2000.3007
26. Gabriel EM, Bergert ER, Grant CS, van Heerden JA, Thompson GB, Morris JC. Germline polymorphism of codon 727 of human thyroid-stimulating hormone receptor is associated with toxic multinodular goiter. *J Clin Endocrinol Metab* (1999) 84:3328–35. doi:10.1210/jcem.84.9.5966
27. Bayram B, Sonmez R, Bozari S, Onlu H, Turkoglu Z, Mutlu FS. The association between development and progression of multinodular goiter and thyroid-stimulating hormone receptor gene D727E and P52T polymorphisms. *Genet Test Mol Biomarkers* (2013) 17:109–14. doi:10.1089/gtmb.2012.0263
28. Muhlberg T, Herrmann K, Joba W, Kirchberger M, Heberling HJ, Heufelder AE. Lack of association of nonautoimmune hyperfunctioning thyroid disorders and a germline polymorphism of codon 727 of the human thyrotropin receptor in a European Caucasian population. *J Clin Endocrinol Metab* (2000) 85:2640–3. doi:10.1210/jcem.85.8.6704
29. Chistiakov DA, Savost'anov KV, Turakulov RI, Petunina N, Balabolkin MI, Nosikov VV. Further studies of genetic susceptibility to Graves' disease in a Russian population. *Med Sci Monit* (2002) 8:CR180–4.
30. Cuddihy RM, Dutton CM, Bahn RS. A polymorphism in the extracellular domain of the thyrotropin receptor is highly associated with autoimmune thyroid disease in females. *Thyroid* (1995) 5:89–95. doi:10.1089/thy.1995.5.89
31. Akamizu T, Sale MM, Rich SS, Hiratani H, Noh JY, Kanamoto N, et al. Association of autoimmune thyroid disease with microsatellite markers for the thyrotropin receptor gene and CTLA-4 in Japanese patients. *Thyroid* (2000) 10:851–8. doi:10.1089/thy.2000.10.851
32. Hiratani H, Bowden DW, Ikegami S, Shirasawa S, Shimizu A, Iwatani Y, et al. Multiple SNPs in intron 7 of thyrotropin receptor are associated with Graves' disease. *J Clin Endocrinol Metab* (2005) 90:2898–903. doi:10.1210/jc.2004-2148
33. C. Wellcome Trust Case Control, C. Australo-Anglo-American Spondylitis, Burton PR, Clayton DG, Cardon LR, Craddock N, et al. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet* (2007) 39:1329–37. doi:10.1038/ng.2007.17
34. Yin X, Latif R, Bahn R, Tomer Y, Davies TF. Influence of the TSH receptor gene on susceptibility to Graves' disease and Graves' ophthalmopathy. *Thyroid* (2008) 18:1201–6. doi:10.1089/thy.2008.0098
35. Ploski R, Brand OJ, Jurecka-Lubieniecka B, Franaszczyk M, Kula D, Krajewski P, et al. Thyroid stimulating hormone receptor (TSHR) intron 1 variants are major risk factors for Graves' disease in three European Caucasian cohorts. *PLoS One* (2010) 5:e15512. doi:10.1371/journal.pone.0015512
36. Chu X, Pan CM, Zhao SX, Liang J, Gao GQ, Zhang XM, et al. A genome-wide association study identifies two new risk loci for Graves' disease. *Nat Genet* (2011) 43:897–901. doi:10.1038/ng.898
37. Colobran R, Armengol Mdel P, Faner R, Gartner M, Tykocinski LO, Lucas A, et al. Association of an SNP with intrathymic transcription of TSHR and Graves' disease: a role for defective thymic tolerance. *Hum Mol Genet* (2011) 20:3415–23. doi:10.1093/hmg/ddr247
38. Liu L, Wu HQ, Wang Q, Zhu YF, Zhang W, Guan LJ, et al. Association between thyroid stimulating hormone receptor gene intron polymorphisms and autoimmune thyroid disease in a Chinese Han population. *Endocr J* (2012) 59:717–23. doi:10.1507/endocrj.EJ12-0024
39. Inoue N, Watanabe M, Katsumata Y, Hidaka Y, Iwatani Y. Different genotypes of a functional polymorphism of the TSHR gene are associated with the development and severity of Graves' and Hashimoto's diseases. *Tissue Antigens* (2013) 82:288–90. doi:10.1111/tan.12190
40. Liu BL, Yang SY, Liu W, Xue LQ, Chen X, Pan CM, et al. Refined association of TSH receptor susceptibility locus to Graves' disease in the Chinese Han population. *Eur J Endocrinol* (2014) 170:109–19. doi:10.1530/EJE-13-0517
41. Bufalo NE, Dos Santos RB, Marcello MA, Piai RP, Secolin R, Romaldini JH, et al. TSHR intronic polymorphisms (rs179247 and rs12885526) and their role in the susceptibility of the Brazilian population to Graves' disease and Graves' ophthalmopathy. *J Endocrinol Invest* (2015) 38:555–61. doi:10.1007/s40618-014-0228-9
42. Fujii A, Inoue N, Watanabe M, Kawakami C, Hidaka Y, Hayashizaki Y, et al. TSHR gene polymorphisms in the enhancer regions are most strongly associated with the development of Graves' disease, especially intractable disease, and of Hashimoto's disease. *Thyroid* (2017) 27:111–9. doi:10.1089/thy.2016.0345
43. Lombardi A, Menconi F, Greenberg D, Concepcion E, Leo M, Rocchi R, et al. Dissecting the genetic susceptibility to Graves' disease in a cohort of patients of Italian origin. *Front Endocrinol* (2016) 7:21. doi:10.3389/fendo.2016.00021
44. Qian X, Xu K, Jia W, Lan L, Zheng X, Yang X, et al. Association between TSHR gene polymorphism and the risk of Graves' disease: a meta-analysis. *J Biomed Res* (2016) 30:466–75. doi:10.7555/JBR.30.20140144
45. Gong J, Jiang SJ, Wang DK, Dong H, Chen G, Fang K, et al. Association of polymorphisms of rs179247 and rs12101255 in thyroid stimulating hormone receptor intron 1 with an increased risk of Graves' disease: a meta-analysis. *J Huazhong Univ Sci Technolog Med Sci* (2016) 36:473–9. doi:10.1007/s11596-016-1611-x
46. Xiong H, Wu M, Yi H, Wang X, Wang Q, Nadirshina S, et al. Genetic associations of the thyroid stimulating hormone receptor gene with Graves diseases and Graves ophthalmopathy: a meta-analysis. *Sci Rep* (2016) 6:30356. doi:10.1038/srep30356
47. Stefan M, Wei C, Lombardi A, Li CW, Concepcion ES, Inabnet WB III, et al. Genetic-epigenetic dysregulation of thymic TSH receptor gene expression triggers thyroid autoimmunity. *Proc Natl Acad Sci U S A* (2014) 111:12562–7. doi:10.1073/pnas.1408821111
48. Tak YG, Farnham PJ. Making sense of GWAS: using epigenomics and genome engineering to understand the functional relevance of SNPs in non-coding regions of the human genome. *Epigenetics Chromatin* (2015) 8:57. doi:10.1186/s13072-015-0050-4
49. Knight JC. Resolving the variable genome and epigenome in human disease. *J Intern Med* (2012) 271:379–91. doi:10.1111/j.1365-2796.2011.02508.x
50. Furey TS, Sethupathy P. Genetics. Genetics driving epigenetics. *Science* (2013) 342:705–6. doi:10.1126/science.1246755
51. McVicker G, van de Geijn B, Degner JF, Cain CE, Banovich NE, Raj A, et al. Identification of genetic variants that affect histone modifications in human cells. *Science* (2013) 342:747–9. doi:10.1126/science.1242429
52. Kilpinen H, Waszak SM, Gschwind AR, Raghav SK, Witwicki RM, Orioli A, et al. Coordinated effects of sequence variation on DNA binding, chromatin structure, and transcription. *Science* (2013) 342:744–7. doi:10.1126/science.1242463
53. Brand OJ, Gough SC. Genetics of thyroid autoimmunity and the role of the TSHR. *Mol Cell Endocrinol* (2010) 322:135–43. doi:10.1016/j.mce.2010.01.013
54. Eisenbarth GS. Type 1 diabetes: molecular, cellular and clinical immunology. *Adv Exp Med Biol* (2004) 552:306–10.
55. Gimenez-Barcons M, Colobran R, Gomez-Pau A, Marin-Sanchez A, Casteras A, Obiols G, et al. Graves' disease TSHR-stimulating antibodies (TSAbs) induce the activation of immature thymocytes: a clue to the riddle of TSAbs generation? *J Immunol* (2015) 194:4199–206. doi:10.4049/jimmunol.1500183
56. Pujol-Borrell R, Gimenez-Barcons M, Marin-Sanchez A, Colobran R. Genetics of Graves' disease: special focus on the role of TSHR gene. *Horm Metab Res* (2015) 47:753–66. doi:10.1055/s-0035-1559646
57. Harmston N, Lenhard B. Chromatin and epigenetic features of long-range gene regulation. *Nucleic Acids Res* (2013) 41:7185–99. doi:10.1093/nar/gkt499
58. Tomer Y, Menconi F. Interferon induced thyroiditis. *Best Pract Res Clin Endocrinol Metab* (2009) 23:703–12. doi:10.1016/j.beem.2009.07.004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 27 September 2016
doi: 10.3389/fendo.2016.00130

TSH Receptor Signaling Abrogation by a Novel Small Molecule

Rauf Latif[1], Ronald B. Realubit[2], Charles Karan[2], Mihaly Mezei[3] and Terry F. Davies[1]*

[1] Thyroid Research Unit, James J. Peters VA Medical Center, Icahn School of Medicine at Mount Sinai, New York, NY, USA, [2] Sulzberger Columbia Genome Center, Columbia University, New York, NY, USA, [3] Department of Pharmacological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Pathological activation of the thyroid-stimulating hormone receptor (TSHR) is caused by thyroid-stimulating antibodies in patients with Graves' disease (GD) or by somatic and rare genomic mutations that enhance constitutive activation of the receptor influencing both G protein and non-G protein signaling. Potential selective small molecule antagonists represent novel therapeutic compounds for abrogation of such abnormal TSHR signaling. In this study, we describe the identification and *in vitro* characterization of a novel small molecule antagonist by high-throughput screening (HTS). The identification of the TSHR antagonist was performed using a transcription-based TSH-inhibition bioassay. TSHR-expressing CHO cells, which also expressed a luciferase-tagged CRE response element, were optimized using bovine TSH as the activator, in a 384 well plate format, which had a *Z* score of 0.3–0.6. Using this HTS assay, we screened a diverse library of ~80,000 compounds at a final concentration of 16.7 μM. The selection criteria for a positive hit were based on a mean signal threshold of ≥50% inhibition of control TSH stimulation. The screening resulted in 450 positive hits giving a hit ratio of 0.56%. A secondary confirmation screen against TSH and forskolin – a post receptor activator of adenylyl cyclase – confirmed one TSHR-specific candidate antagonist molecule (named VA-K-14). This lead molecule had an IC_{50} of 12.3 μM and a unique chemical structure. A parallel analysis for cell viability indicated that the lead inhibitor was non-cytotoxic at its effective concentrations. *In silico* docking studies performed using a TSHR transmembrane model showed the hydrophobic contact locations and the possible mode of inhibition of TSHR signaling. Furthermore, this molecule was capable of inhibiting TSHR stimulation by GD patient sera and monoclonal-stimulating TSHR antibodies. In conclusion, we report the identification of a novel small molecule TSHR inhibitor, which has the potential to be developed as a therapeutic antagonist for abrogation of TSHR signaling by TSHR autoantibodies in GD.

Keywords: TSH receptor, small molecule, antagonist

OPEN ACCESS

Edited by:
Alessandro Antonelli, University of Pisa, Italy

Reviewed by:
Salvatore Benvenga, University of Messina, Italy
Salvatore Ulisse, Sapienza University of Rome, Italy

****Correspondence:***
Rauf Latif
rauf.latif@mssm.edu

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *22 July 2016*
Accepted: *06 September 2016*
Published: *27 September 2016*

Citation:
Latif R, Realubit RB, Karan C, Mezei M and Davies TF (2016) TSH Receptor Signaling Abrogation by a Novel Small Molecule. Front. Endocrinol. 7:130. doi: 10.3389/fendo.2016.00130

Abbreviations: BD, Brownian dynamics; FSH, follicle-stimulating hormone; FSK, forskolin; HTS, high-throughput screening; LH, luteinizing hormone; MD, molecular dynamics; SML, small molecule ligands; TMH, transmembrane helix; TSHR, TSH receptor.

INTRODUCTION

The TSH receptor (TSHR) is primarily expressed in the basolateral surface of thyroid follicular cells and induces thyroid cell growth, hormone synthesis, and hormone secretion and also happens to be a primary autoantigen in autoimmune thyroid disease; especially Graves' disease (GD) (1–4). GD is one of the most common organ-specific autoimmune diseases with a prevalence of ~2% in the general population (5). It is an antibody and T cell-mediated disease where hyperstimulation of the thyroid gland leads to excess thyroid hormone production. The pathogenic effects of GD are driven, in part, by the interaction of stimulating antibodies to the TSHR, which bind to its large extracellular domain (ECD) (6). Such autoantibodies come in different varieties that can stimulate, block, or lead to apoptosis *via* induction of cellular stress (2, 7). In addition to its primary site on the thyroid cell, the TSHR is also expressed in a variety of extra thyroidal tissues where it is known to modulate target cell function, including fibroblasts and adipocytes and osteoclasts and osteoblasts (8–13). For example, there is evidence for a role of the TSHR in Graves' orbitopathy and retro-orbital adipogenesis (13, 14) and as a negative regulator in bone remodeling (11). The presence of the TSHR in these and other extra thyroidal depots (10) makes it an important candidate receptor for several undefined roles secondary to the cascade of effects that may result from its chronic stimulation in GD.

In the last few years, small molecules have gained momentum as therapeutic options secondary to the development of large chemical libraries and robust high-throughput screening (HTS) assays (15). In addition to their low cost and ease to manufacture, they also have inherent chemical and biological advantages. These advantages include their ease in crossing plasma membrane barriers and their *in vivo* stability due to their resistance to proteolytic enzymes. Small molecule agonists against the TSHR have been reported by others (16, 17), as well as ourselves (18). However, to date, only a single TSHR antagonist has been reported, which was found following chemical modification of an agonist, but its potency is only in the micro molar range (19). There is now a need to improve the potency of such molecules to achieve a therapeutic IC_{50} in the nano molar range (10^{-9}M).

All small molecules interacting with the TSHR appear to permeate the cell and dock with distinct polar and non-polar residues within the hydrophobic pockets created by the helices of the transmembrane (TM) domain and exert a stimulatory or inhibitory effect by altering the interaction and movement of these helices (20, 21), thus acting as novel pharmacophores. This report describes the identification and *in vitro* characterization of a small molecule antagonist to the TSHR selected by a chemical library screen using an in-house luciferase-based high-throughput inhibition assay.

MATERIALS AND METHODS

Materials

Bovine TSH (1 IU/ml), human FSH (70 IU/ml), hCG (10 IU/vial), and forskolin (FSK) were purchased from Sigma-Aldrich (St Louis, MO, USA). The Bright-Glo™ luciferase substrate (Cat # E2610) was purchased from Promega Corporation, Madison, WI, USA. The cell culture medium, DMEM, and Ham's F12 were purchased from Mediatech Inc., Manassas, VA, USA. Fetal bovine serum and fetal calf serum were purchased from Atlanta Biologicals, Flowery Branch, GA, USA. Additional amounts of lead compounds that were identified by screening were purchased from Enamine Inc., Cincinnati, OH, USA.

Screening Libraries

Three libraries were used in the screening: (1) Lead-Optimized Compound library (LOC) made up of 9,690 molecules, (2) Enamine library made of 60,638 molecules, and (3) Analyticon library made up of 10,000 molecules. All three libraries were specifically designed by the Columbia University HTS facility (22, 23). A total of 80,328 molecules were screened as a single point, at a concentration of 16.7 μM. All potential hits were than analyzed by dose–response studies in triplicate.

Cell Lines Used

(a) *CHO-HA-TSHR luciferase cells*: For HTS, we used cells generated by transfecting the pGL4.29 [luc2P/CRE/Hygro] construct into a highly selected stable line of CHO cells expressing the human TSH receptor with an hemagglutinin (HA) tag at the *N*-terminus (CHO-HA-TSHR cells) that has been previously described and was selected as a stable line with hygromycin (18). The cells were cultured in Ham's F-12 medium with 10% fetal bovine serum (FBS) and 100 IU/ml of penicillin, 100 μg/ml streptomycin, and 50 μg/ml of hygromycin.

(b) *Murine Sertoli cell line TM4*: These FSH receptor expressing cells were obtained from ATCC (CRL-1715) and cultured in DMEM: F12 medium (cat # 30-2006) with 2.5% FBS and 5% horse serum (ATCC; cat #30-2040).

(c) *LH receptor-expressing cells*: The specificity against the LH/hCG receptor was tested using a stable line of rat LH/hCG receptor expressing HEK 293 cells that were kindly provided by Dr. K. M. J. Menon, University of Michigan, Ann Arbor, MI, USA. These cells were cultured in DMEM medium with 10% FBS and 100 IU/ml of penicillin and 100 μg/ml streptomycin.

HTS Inhibition Assay

This assay was based on the same principle as described previously (18) for screening of agonist molecules. Briefly, 15,000 CHO-HATSHR Luci #1 cells [named TSHR-Glo cells (15)] were plated into white standard 384 wells at a volume of 30 μl in Ham F12 complete medium and incubated overnight at 37°C at >85% humidity. Library compounds were added at 16.7 μM to each well using a 384 nano-head (Perkin Elmer Inc.) and preincubated for 1 h at 37°C prior to stimulant addition. The compound added wells were then stimulated with 5 μl corresponding to 20 μU of bovine TSH for 4 h. To determine the luciferase activity in these cells at the end of incubation, the wells received 13 μl of the substrate Bright-Glo™. The luminescence was then measured using an EnVision multilabel reader (Perkin Elmer Inc.). In principle, activation of the TSHR by TSH results in $G_{s\alpha}$-adenylate cyclase coupling and an increase in intracellular cAMP, which results in

the activation of CREB and its binding to the CRE element and subsequently induces the transcription of the *luciferase* gene and accumulation of the luciferase enzyme within the activated cells. Since the cells are preincubated with compounds that may inhibit the activation of Gsα-adenylate cyclase system, TSH activation of the receptor would be inhibited if the compound is a specific TSHR antagonist. However, the screen may result in false positives that inhibit activation of adenylate cyclase directly and thus inhibiting cAMP generation. Therefore, hits that are picked up in an initial screen must, then, be tested against FSK to rule out such false positives.

Throughout the screen, the signal to background ratio was linear and the mean CV was 5.4% and the Z′ factor was in the range of 0.3–0.6 based on the positive control Antag3 (19) (also kindly provided by Dr. M. Gershengorn, NIH, Bethesda, MD, USA) used in the plate. This exceeded the commonly accepted threshold for validation of high-throughput assays (24). When we challenged the cells with two different concentrations of bovine TSH (10 and 100 μU) (**Figure 1A**), we found that stimulation with 10 μU of TSH gave an inhibition of ~30–40% compared with less than 10% inhibition observed by stimulation with 100 μU of TSH. However, on optimization of the HTS, we found 20 μU TSH as the best stimulation because it gave similar inhibition in the HTS. We used an arbitrary fixed criteria for selecting molecules as positive hits if they showed ≥50% inhibition of TSH activity. Medium with <1% of DMSO was the negative control, whereas the control molecule with TSH and just TSH alone acted as positives in the assay for normalization of the signal and identification of positives hits.

Confirmatory Assays

Dose–responses of the lead molecules were performed against TSH and FSK using a Tecan HP digital dispenser by following a similar protocol as described. All data points of the dose–response curves were fitted using Prism 5.0. A fluorescent viability assay (Cell Titer-Fluorviability assay, Promega Inc.) was also performed in the same experiment to assess toxicity of the molecules.

Docking of Lead Molecule on the TSHR Transmembrane Domain

Docking of the lead molecules was performed on a homology model of the TSHR-TMD based on rhodopsin (PDB:1F88). This template was chosen because of the low root-mean-square deviation (RMSD) values between the backbone of the TM helices of the TSHR model and that of the rhodopsin X-ray crystal structure (25) and fits the experimental parameters that we have previously described (26). The initial homology model of rhodopsin was obtained from the Uniprot server.[1] The conformations of the extracellular loops were constructed with the Monte Carlo method (27). The 3D geometries of the molecules in Tripos'mol2 format were generated with MarvinSketch.[2] Docking was carried out using the docking, Autodock 4. The docking results were analyzed using DOCKRES and other supporting script tools (28).

Serum Samples

Serum samples used in this study were unidentifiable stored samples originally collected with the full consent of patients.

Statistical Analyses

All curve fitting and EC_{50} calculations were performed using GraphPad Prism version 5.02, and statistical differences for *P* values were calculated using one-tailed *t* test using Graph Pad In Stat software.

RESULTS

Evaluation of the HTS Luciferase-Based Inhibition Assay for Screening and Identification of TSHR-Specific Inhibitors

Using this HTS inhibition assay, we screened a total of 80,328 molecules consisting of all three libraries as described earlier at a

[1]http://www.uniprot.org
[2]http://www.chemaxon.com

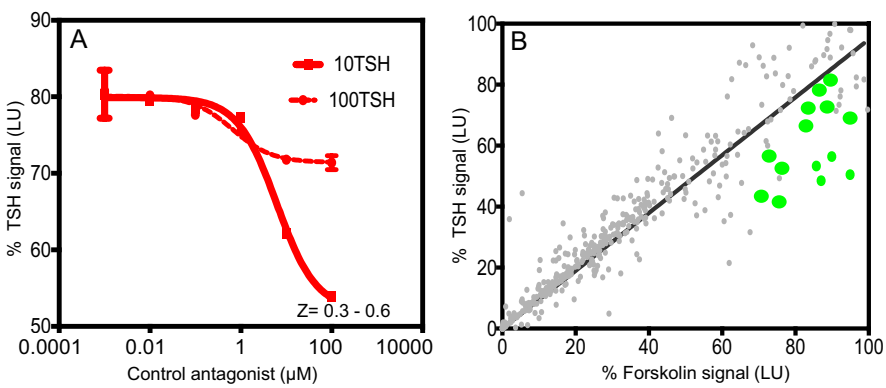


FIGURE 1 | Inhibition assay for detection of TSHR antagonists and overview of positive hits. (A) The HTS luciferase assay that was developed for detection of antagonists against the TSHR was first tested for its TSH inhibitory activity using bovine TSH (10 and 100 μU) against a control antagonist. We screened a diverse chemical library of ~80,000 compounds for novel antagonists using this assay. **(B)** All positive hits were tested in parallel against TSH (*y*-axis) and forskolin (*x*-axis) as shown in this *x*–*y* plot. Molecules that showed >50% inhibition of TSH signal but 0 or <5% inhibition of forskolin signal were considered as potential inhibitors of the TSHR. Fourteen such randomly selected representative molecules are shown as green dots on this plot.

single concentration of 16.7 μM. We obtained 450 positives hits from this initial screen with a hit ratio of 0.56%. The performance of the assay throughout the screen is indicated by the *Z* score, signal to background ratio, and % CV plots (**Figure 2**). Further, to eliminate false positives and to obtain a secondary confirmation of the positives, we performed a secondary testing at 16.7 μM against 20 μU of TSH and 10 μM of FSK. **Figure 1B** is an *x*–*y* plot showing the results of such a screen where percent of TSH luciferase signal is indicated in the *y*-axis with percent of FSK signal in the *x*-axis. Molecules that showed 50% or greater inhibition of TSH and none or very little inhibition against FSK were generally regarded as potential inhibitory molecules specific to the TSHR and marked out for dose–response analysis. Fourteen such potential hits (marked by green circles) in **Figure 1B** are represented in the plot.

Selection of a Specific TSHR Antagonist

Using our selection criteria, we identified 14 molecules as potential inhibitory molecules. These 14 molecules were further tested in triplicate at 16.7 μM against stimulation with 20 μU of TSH and 10 μM of FSK to confirm their specific inhibitory potency as indicated (**Figure 3**). Three molecules (marked by the arrows) appeared to have potential and were subjected to dose–response studies against TSH and FSK.

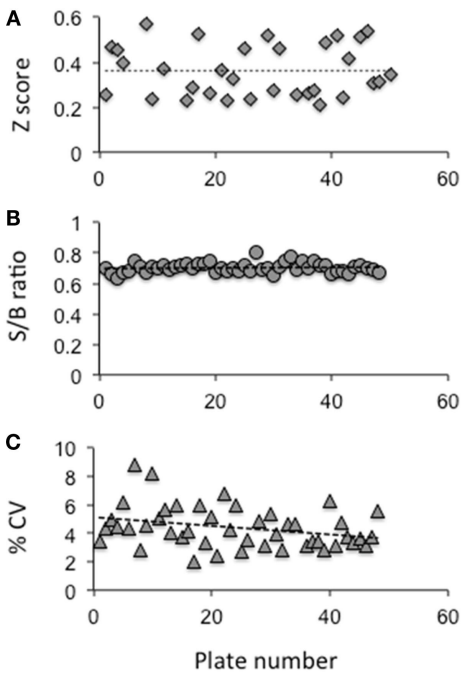


FIGURE 2 | **Evaluation of the HTS assay**. **(A)** The *Z* factor was calculated using the positive control and basal control responses in each plate as per the formula described by Zhang et al (24) **(B)** The signal to background ratio was obtained using the total signal from the positive control well against those wells receiving medium plus DMSO. **(C)** The coefficient of variation (% CV) was calculated as the SD from the wells with the basal medium divided by the wells of the positive control. The data indicated that the HTS assay performed within the limits of a reliable screening.

Dose–responses of each of the selected molecules (K14, L13 and F9) are represented in the different panels in **Figure 4** along with one non-specific molecule (D22). The dose–response curves of the molecules strongly indicated that K14 had a 30–40% inhibition of TSH with negligible inhibition of FSK and low cytotoxicity compared with molecules L13, F9, or D22. Since K14 showed specific inhibition in the range examined, this was regarded as our most specific lead antagonist against the TSHR (now referred to as VA-K-14). VA-K-14 had an $IC_{50} = 12.32$ μM, and, although it was specific to the TSHR, it appeared to have a narrow inhibitory range.

Specificity Analysis of VA-K-14

We, next, analyzed the specificity of VA-K-14 against other closely homologous glycoprotein receptors – the FSH receptor and LH/hCG receptor – using a cAMP femto HTRF bioassay (Cat # 62AM5PEB, Cisbio Inc.). For the LH receptor cells, we used HEK 293 cells transfected with the rat LH/hCG receptor, and, for the FSH receptor, we used a murine Sertoli cell line (TM4), which expresses the FSHR and responds to human FSH in a dose-dependent manner. Inhibition of intracellular cAMP generation was measured after stimulation of these cells with maximal responsive doses of their respective ligands (TSH, FSH, and hCG), after preincubation with VA-K-14 (0.01–100 μM). The TSHR-CHO cells were stimulated with 20 μU of bovine TSH, LH/hCC receptor cells with 1000 μU/ml of hCG, and Sertoli cells were stimulated with 700 μU/ml of human FSH, which had previously been titrated for optimum stimulation of cAMP under our experimental conditions (18). VA-K-14 showed more than 40% inhibition on the TSHR-expressing cells (**Figure 5A**). VA-K-14 showed a minor degree of inhibition (~10–15%) against the hCG/LH and FSH receptor-expressing cells, suggesting small molecules that are strong antagonists against the TSHR might have inhibitory effects against their homologous glycoprotein hormone receptors as seen previously (19).

Define the Binding Sites by Molecular Docking

Inhibitory small molecule ligands are usually allosteric modulators of GPCRs (29). **Figure 5B** shows the molecular structure of VA-K-14 – which is *N*-methyl-4-(2-phenyl-1H-indol-3-yl)-thiazole-2-amine with a molecular weight of 305.406 Da. This molecule meets the Lipinski rule-of-five criteria (30) with an *x*log *P* of 4.34 and tPSA of 40. It is dissimilar in structure to the one published TSHR antagonist (19).

We examined the binding sites of VA-K-14 to the TM region of the TSHR by *in silico* docking, using a structure of the TSHR-TM region developed in our laboratory by homology modeling based on the rhodopsin crystal structure (26) and Monte Carlo simulations. By examining the top scoring docking poses generated by Autodock 4 and Autodock-Vina (all clustered at the same region of the extracellular hydrophobic pocket), we were able to deduce the putative contact residues within the TSHR-TM domain (**Figure 5C**). Docking analysis indicated that VA-K-14 is likely to make contact with residues Asn 483 (N483) and Trp 488 (W488) in ECL1 and Leu 468 (L468) on TMH1, Thr500 (T500) in TMH3,

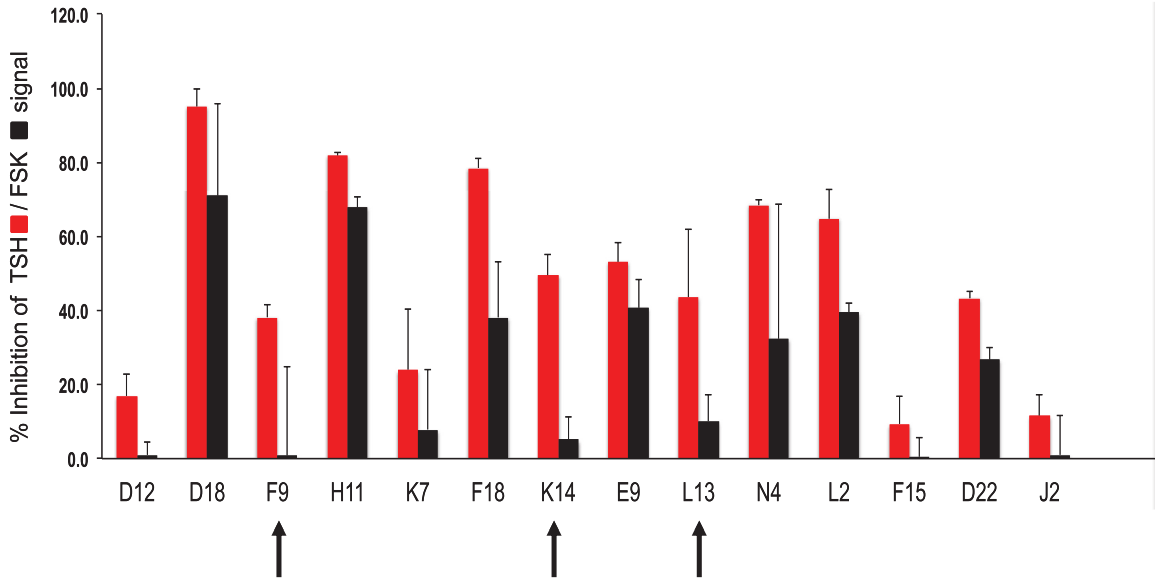


FIGURE 3 | **Hit validation**. This bar graph illustrates the testing of the fourteen chosen compounds for inhibition of TSH and forskolin (FSK) signaling (mean ± SEM). The percent inhibition of the maximum TSH or forskolin signal is represented on the *y*-axis. Indicated by the arrows were three potentially specific candidates that showed minimal inhibition of forskolin (black bars) but significant inhibition of TSH (red bars). These three candidate molecules were then subjected to dose–response analyses.

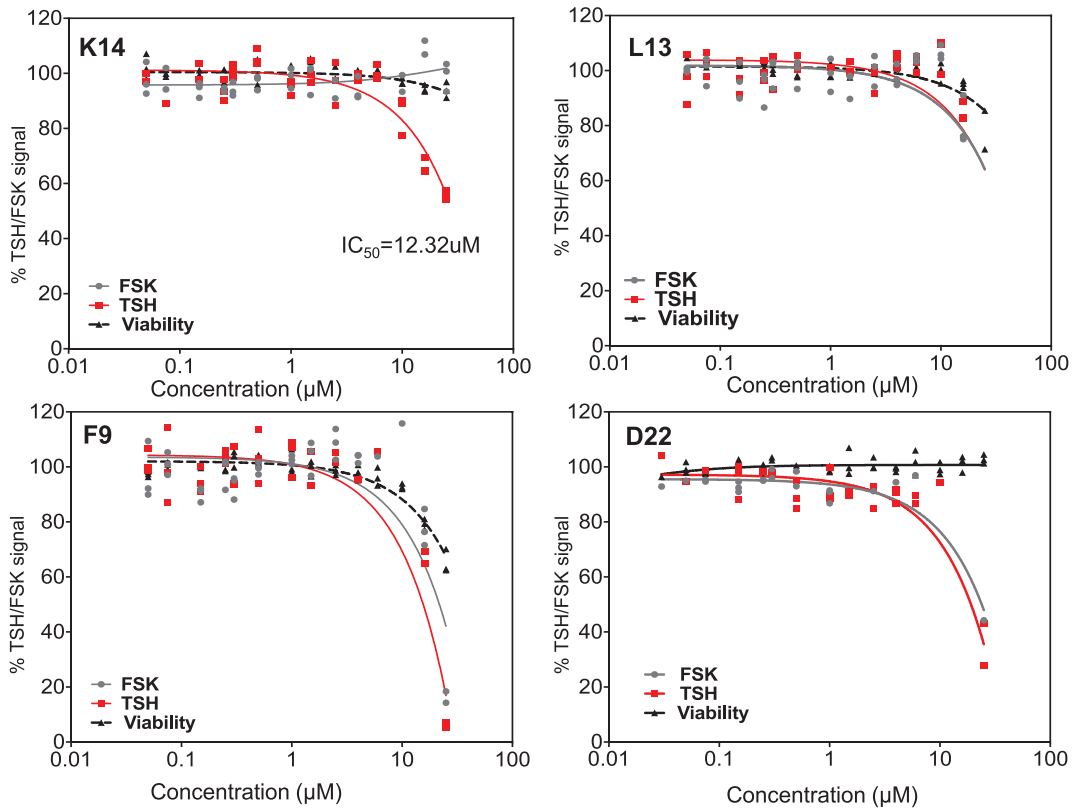


FIGURE 4 | **Testing of selected lead molecules**. These four panels indicate the dose–responses of three likely (from **Figures 3** and **1**) and one control unlikely lead molecule against TSH (red) and FSK (gray). VA-K14 was the only molecule that effectively inhibited the TSH signal and had no inhibition for FSK compared with L13, F9, and D22. The toxicity of these molecules was tested by measuring viability (black) using the Cell titer Fluor assay within the same assay.

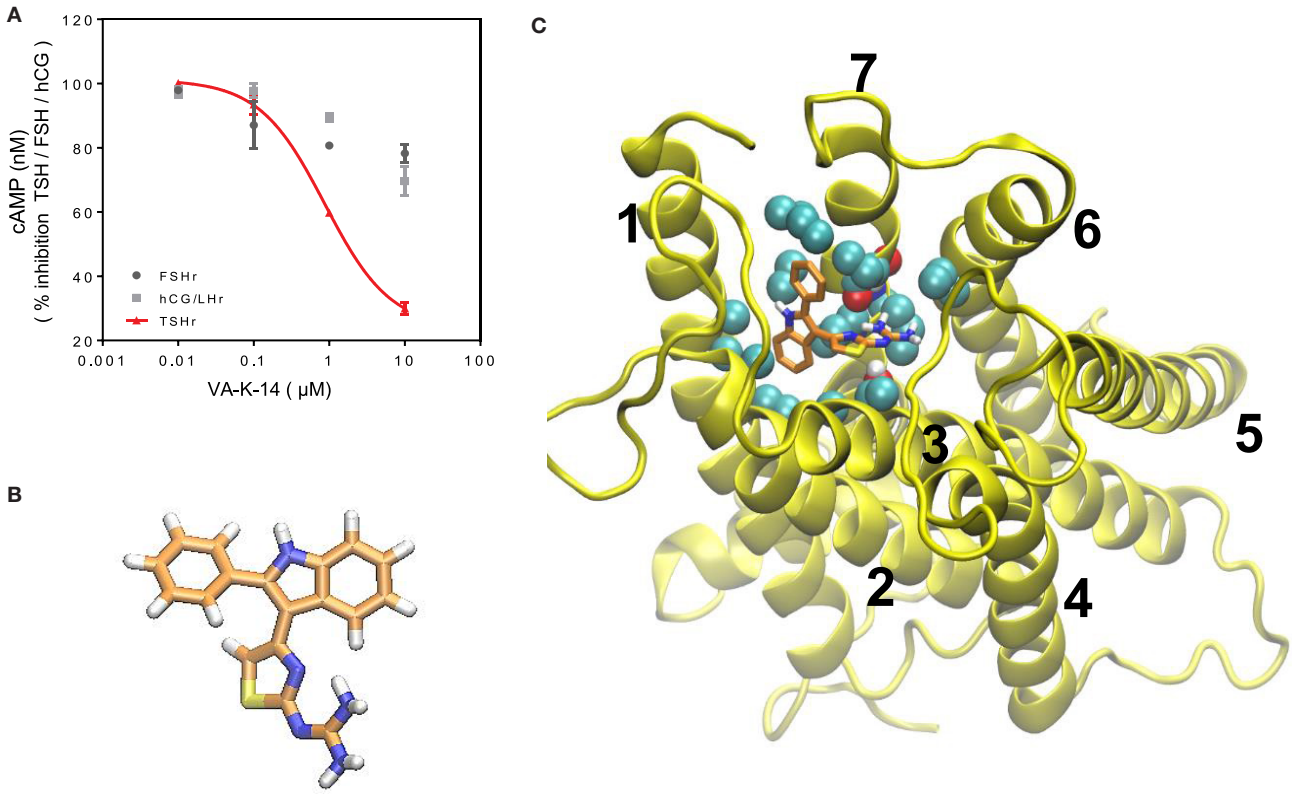


FIGURE 5 | **(A)** Specificity and TSHR docking of the lead molecule **(A)** CHO-TSHr stable cells, HEK-LH/CGr stable cells, and FSHr- Sertoli cells (TM4) were stimulated with the maximum effective concentrations of their respective ligands as described in Section "Materials and Methods" for 1 h at 37°C in the presence of 1 mM IBMX and increasing concentrations of our lead antagonist VA-K-14. As indicated by the red line, VA-K-14 inhibited TSH by 75% at 10 μM in contrast to an inhibition of 10–15% with the hCG/LHr cells and FSHr cells. These data were average plots of two independent experiments performed in triplicate. **(B)** The structure of VA-K-14 is *N*-methyl-4-(2-phenyl-1H-indol-3-yl)-thiazole-2-amine with a molecular weight of 305.406 Da. **(C)** *In silico* docking performed on the homology model of the TSHR transmembrane domain (26) using Autodock 4 strongly suggested that VA-K-14 docks into the hydrophobic pocket of the TSHR–TMD, thus making contact with two residues in extracellular loop 1 (ECL1) (residues Asparagine 483 and tryptophan 488) and further contacts with Leucine 468 on TMH 2, Threonine 500 on TMH 3, and Valine 664 on TMH 7.

and Val 664 (V664) in TMH7 within the previously described hydrophobic pocket of the TMD (20, 21).

Inhibition of TSHR Antibodies

We first tested our luciferase assay for inhibition of signal response in the presence of potent blocking TSHR antibody that binds to the ectodomain of the receptor. **Figure 6A** shows the inhibition of luciferase signal observed on stimulation of cells with 50 μU of TSH in the absence of antibody (gray bar) and in the presence of increasing doses of a monoclonal human TSHR blocking antibody (K1-70) (31) (kindly provided by Dr Bernard Rees Smith, RSR Ltd., Cardiff, Wales). Nearly 40% inhibition of stimulation was observed at 10 μg of the K1-70 blocking antibody.

In order to assess if our lead antagonist VA-K-14 was capable of inhibiting stimulating TSHR antibodies in GD patient sera, we tested a panel of 14 Graves' sera (diluted 1:10) in the absence and presence of 10 μM of VA-K-14. We observed variable degrees of inhibition of stimulation although all the sera were inhibited to some degree (**Figure 6B**). VAK-14 was also effective in inhibiting a widely used human monoclonal-stimulating antibody [M22, also kindly provided by Dr Bernard Rees Smith, RSR Ltd., Cardiff, Wales (32)] and a hamster-derived stimulating monoclonal antibody (MS-1) (33) (**Table 1**).

Combining Two Structurally Dissimilar Antagonists

When we compared VA-K-14 with the control antagonist (Antag 3), which is structurally dissimilar and makes contact with disparate residues in the TMD region, we found similar degrees of inhibition at concentrations between 1 and 100 μM, but comparison of the area under the curves (AUC) showed that the two molecules were significantly ($P = 0.003$) different in their degree of inhibition (**Figure 7A**). This significant difference in their dose–response relationships also most likely indicated their binding to different residues in the TSHR-TM domain. Since VA-K-14 and Antag 3 have different binding sites within the hydrophobic pocket of the receptor, we examined the effect on inhibition of stimulation by combining the two. This analysis clearly indicated that combining two antagonists did not enhance the degree of inhibition (data not shown), suggesting that the complex dynamic molecular interactions that the small molecules make with the receptor allosteric site has limits to its distortion potential.

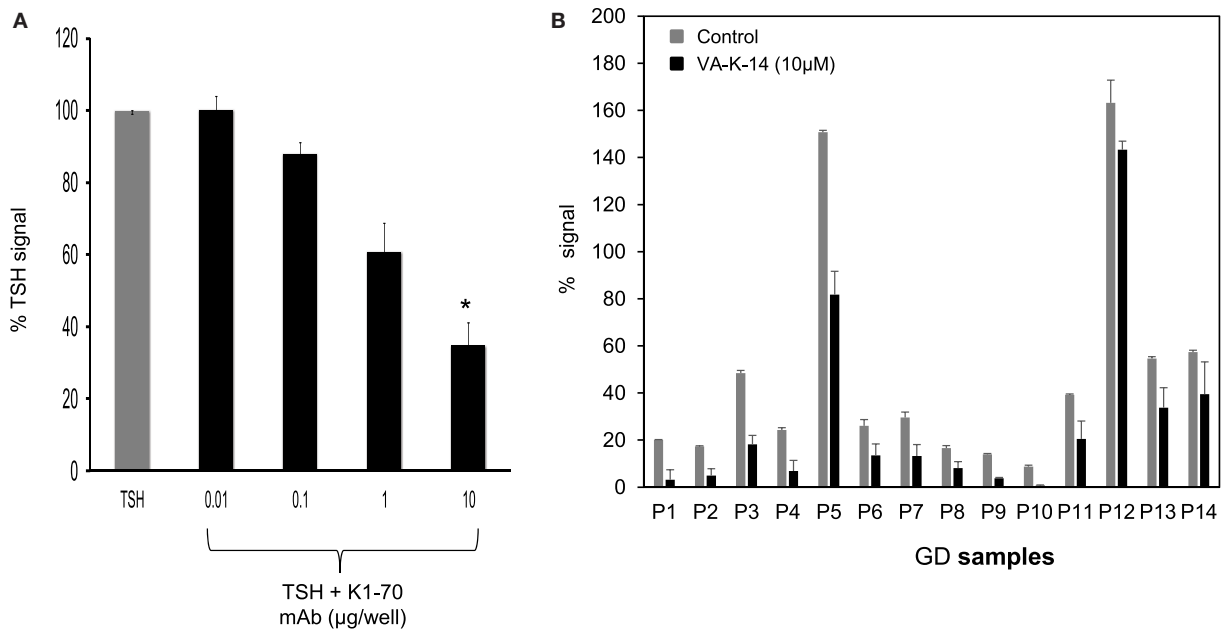


FIGURE 6 | Inhibition of GD sera by VA-K-14. **(A)** The bar graph shows the inhibition of TSH signal (50 µU/ml) by a human blocking monoclonal antibody (K1-70). Increasing doses of antibody caused inhibition of TSH signal. Significant inhibition ($P = 0.0116$) was observed at 10 µg/ml of K1-70 monoclonal antibody. This suggested that the inhibition assay was capable of measuring TSHR-Ab inhibition of cAMP generation. **(B)** We tested a series of GD serum samples at 1:10 dilution for inhibition by VA-K-14. The cells were first preincubated with 10 µM of VA-K-14 or just medium and then challenged with diluted serum in triplicate wells. As indicated here, there was a varied degree of inhibition observed in the presence of 10 µM of VA-K-14 (black filled bars) compared with untreated serum (gray bars) with the luciferase assay. As seen here, P3 and P5 showed the most significant suppression of their stimulating responses in the presence of antagonist, but inhibition of P12 was poor.

TABLE 1 | Dose-dependent inhibition of M22 and MS1 by molecule VA-K-14.

Dose of VA-K-14 (µM)	M22 (% change)	MS1 (% change)
0	100	100
0.1	99.93 ± 2.36	95.64 ± 4.56
1	83.63 ± 3.38	90.73 ± 0.91
10	68.36 ± 0.49	64.47 ± 1.00

Note: the dose of M22 used for stimulation was 1.5 µg final and that of MS-1 was 15 µg, final. The assay was carried out with 15,000 cells/well in 348 well plate using CHOTSHR luciferase cells.

Mechanism of VA-K-14 Action

Docking analysis indicated that VA-K-14 makes contact with tryptophan 488 (W488) on ECL1 and a second contact with Valine 664 (V664) in TMH 7 by launching itself in the hydrophobic pocket formed by helices of the TSHR TMD (**Figure 7B**). It is known that W488 is an important residue of a naturally occurring inactivating mutation (34) and V664 of helix 7 is a critical partner with Isoleucine 568 (35) on ECL2 which helps in stabilization of the receptor conformation.

DISCUSSION

Hyperthyroid GD is currently treated with antithyroid drugs, radioactive iodine, or surgery (36). However, these modes of treatment though effective are not without complications and antithyroid drug treatment is commonly followed by relapses (37, 38). Antithyroid drugs can also cause severe or even life-threatening complications. Nevertheless, there have been few attempts to develop newer drugs that would be more effective. Small molecule antagonists that can inhibit signaling of the TSHR do, however, have serious potential as novel therapeutic options. We have previously reported the development of two lead small molecule agonists against the TSH receptor (18). In this study, we describe the *in vitro* characterization of a novel TSHR antagonist identified by high-throughput screening using a transcriptional-based luciferase inhibition assay.

Though the TSHR is promiscuous in engaging several G proteins (39) and β-arrestin 1 and 2 (40–42), it is known that the predominant signal that comes from the receptor is *via* engagement of the Gs subclass of G protein, which leads to the generation of second messenger cAMP. We have previously exploited this major signaling pathway to develop a sensitive HTS assay for TSHR agonist detection, known as the TSHR-Glo assay (15). We extended the stimulation assay by first pre-incubating the cells with the library compounds and, then, stimulating them with an optimized dose of bovine TSH for 4 h before reading the luciferase signal. Though the inhibition HTS assay was not robust in its performance as just judged by *Z* factor score, it was effective enough to detect 30–40% inhibition of low dose TSH stimulation and maintained a fairly consistent *Z* score (**Figures 1A** and **2**). However, on screening a diverse library of compounds of 80,328 molecules using this HTS assay, we identified only one lead molecule (VA-K-14) that was specific to the TSHR and failed to show

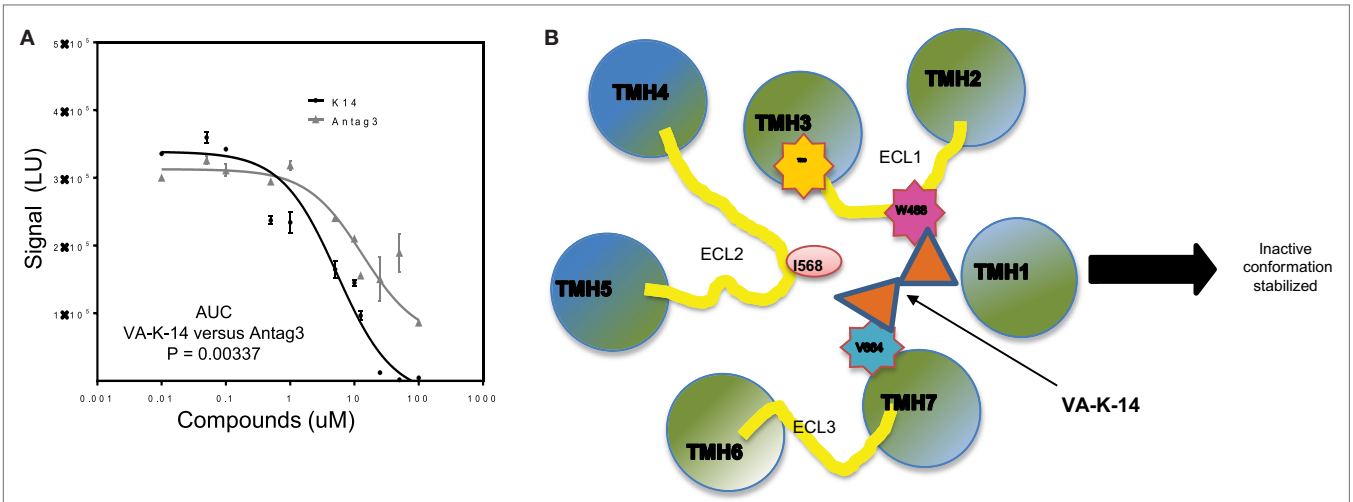


FIGURE 7 | **Mechanistic action of VA-K-14**. **(A)** Indicated in this dose–response graph is a comparison of our lead molecule VA-K-14 and the reported Antag3 in order to compare their inhibition of TSH signaling. Although the two molecules are structurally different, they clearly have the potential to inhibit TSH action in a similar manner. However, the area under the curve calculation of VA-K-14 versus Antag3 indicated VA-K-14 to be significantly different in its inhibitory characteristics of the TSH response in this assay. **(B)** This diagram indicates the possible mechanism for the inhibitory effect of VA-K-14 on binding to the hydrophobic pocket in the TMD of the TSHR. The molecule contacts tryptophan 488 (W488) in ECL1 (pink) and valine 664 (V664) on helix 7 (blue). W488 is a naturally occurring inactivating mutation where V664 is a critical partner with Isoleucine 568 in ELC2 in stabilization of the receptor conformation. Thus, it would seem that this charge interaction network formed by VA-K-14 in the hydrophobic pocket must play a role in stabilizing an inactive conformation of the receptor transmembrane and, thus, leading to dampening of the signal.

any inhibition of FSK – a post receptor activator of cAMP – even in dose–response analysis (**Figure 4**).

Since the TM domain of major GPCR's, especially the glycoprotein hormone receptor family, such as the FSH and hCG/LH receptors, is quiet homologous (43) in their sequence, it was important to examine receptor specificity of the lead small molecule. Using cells that express the FSH and LH receptor cells, we examined the specificity of our lead molecule VA-K-14. Though a potent inhibitor of TSHR, it also showed minor inhibition of cAMP generation against FSH and LH receptor bearing cells at the highest effective concentrations as seen with the previously reported antagonist (Antag 3) (19). The homologous nature of the TM domain of these receptors may cause such reactivity to be inevitable, and a potent antagonist, which was reported previously to be in the nanomolar range (44), was also found to lack all specificity against the TSHR and, thus, could not be developed further. But, it is known that functional ability of the allosteric modulators against the TSHR are defined by the contact residues within the TM domain (20), and the pharmacophore property of small molecules can be altered by structural alteration of their scaffold as evidenced by the development of Antag 3 (19).

To examine the contact sites of our lead molecule (VA-K-14), we docked the molecule with a well verified TM domain structural model that we previously developed on the rhodopsin template (26). From the docking studies, it was clear that VA-K-14 docked in the hydrophobic pocket of the TSHR-TM domain making polar and non-polar contacts with residues asparagine 483 (N483) and tryptophan 488 (W488) on the extracellular loop 1 (ECL1) and leucine 468 (I468) on TM 2, threonine 500 (T500) on TM 3, and valine 664 (V664) in TM 7. The contact residues of this molecule in the hydrophobic pocket are different from those of our lead agonists (18) and were also different from Antag 3.

It is known that GPCR activation could involve the movement of helices, especially TM3 and TM6 (45–47), and modeling studies with mutational analyses have clearly outlined several residues within the hydrophobic pocket defined by the various helices of the TMD (20, 21, 48). Hence, the molecular property of allosteric small molecules will reside in the mosaic of interactions that such a molecule makes within the pocket, thus stabilizing an active or inactive state of the TSHR. It is known that residue W488 in ECL1 is a naturally occurring inactivating mutation (34) and V664 on TMH7 is a critical partner with I568 in ECL in stabilizing a native receptor conformation (35). Thus, from the docking data, we can hypothesize that VA-K-14, by interacting with these two residues, may stabilize an inactive form of the TSHR, leading to inhibition of TSH signaling. Furthermore, a recent study (49) using computation analysis and chemical crosslinking followed by mass spectrometry has also shown that rearrangement of the ECD/ECL1 is critical for TSHR activation. Thus, any allosteric molecule that could thwart such a rearrangement (50) would be a potential negative allosteric modulator (NAM).

In conclusion, we identified and characterized, *in vitro*, a new antagonist of the TSHR, which is a potential candidate for further development to provide a therapeutic option for controlling the action of auto antibodies to the TSHR in hyperthyroid GD and its related manifestations.

AUTHOR CONTRIBUTIONS

RL: assay development and majority of the experiments and wrote manuscript. RR: did the screening of molecules. CK: coordinated the screening and data analysis. MM: did the docking work. TD: coordinate the project, data analysis and writing of manuscript.

ACKNOWLEDGMENTS

The authors thank Drs. R. Baliram, Risheng Ma, and Syed A Morshed for critical reading of the manuscript and their comments. The authors also wish to thank Miss Xhensila Hyka from Sulzberger Columbia Genome Center, Columbia University for her technical assistance. This work was supported in part by NIH grant DK069713 & the VA Merit Award BX000800 (to TD).

REFERENCES

1. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM. The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. *Endocr Rev* (1998) 19:673–716. doi:10.1210/edrv.19.6.0352
2. Davies TF, Ando T, Lin RY, Tomer Y, Latif R. Thyrotropin receptor-associated diseases: from adenomata to Graves disease. *J Clin Invest* (2005) 115:1972–83. doi:10.1172/JCI26031
3. Vassart G, Dumont JE. The thyrotropin receptor and the regulation of thyrocyte function and growth. *Endocr Rev* (1992) 13:596–611. doi:10.1210/er.13.3.596
4. Smith BR, Sanders J, Furmaniak J. TSH receptor antibodies. *Thyroid* (2007) 17:923–38. doi:10.1089/thy.2007.0239
5. Boelaert K. Treatment of Graves' disease with antithyroid drugs: current perspectives. *Thyroid* (2010) 20:943–6. doi:10.1089/thy.2010.1654
6. Furmaniak J, Sanders J, Nunez Miguel R, Rees Smith B. Mechanisms of action of TSHR autoantibodies. *Horm Metab Res* (2015) 47:735–52. doi:10.1055/s-0035-1559648
7. Morshed SA, Ma R, Latif R, Davies TF. How one TSH receptor antibody induces thyrocyte proliferation while another induces apoptosis. *J Autoimmun* (2013) 47:17–24. doi:10.1016/j.jaut.2013.07.009
8. Atkins SJ, Lentz SI, Fernando R, Smith TJ. Disrupted TSH receptor expression in female mouse lung fibroblasts alters subcellular IGF-1 receptor distribution. *Endocrinology* (2015) 156:4731–40. doi:10.1210/en.2015-1464
9. Smith TJ, Padovani-Claudio DA, Lu Y, Raychaudhuri N, Fernando R, Atkins S, et al. Fibroblasts expressing the thyrotropin receptor overarch thyroid and orbit in Graves' disease. *J Clin Endocrinol Metab* (2011) 96:3827–37. doi:10.1210/jc.2011-1249
10. Davies T, Marians R, Latif R. The TSH receptor reveals itself. *J Clin Invest* (2002) 110:161–4. doi:10.1172/JCI0216234
11. Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y, et al. TSH is a negative regulator of skeletal remodeling. *Cell* (2003) 115:151–62. doi:10.1016/S0092-8674(03)00771-2
12. Baliram R, Sun L, Cao J, Li J, Latif R, Huber AK, et al. Hyperthyroid-associated osteoporosis is exacerbated by the loss of TSH signaling. *J Clin Invest* (2012) 122:3737–41. doi:10.1172/JCI63948
13. Kumar S, Coenen MJ, Scherer PE, Bahn RS. Evidence for enhanced adipogenesis in the orbits of patients with Graves' ophthalmopathy. *J Clin Endocrinol Metab* (2004) 89:930–5. doi:10.1210/jc.2003-031427
14. Bahn RS. Current insights into the pathogenesis of Graves' ophthalmopathy. *Horm Metab Res* (2015) 47:773–8. doi:10.1055/s-0035-1555762
15. Latif R, Lau Z, Cheung P, Felsenfeld DP, Davies TF. The "TSH receptor glo assay" – a high-throughput detection system for thyroid stimulation. *Front Endocrinol* (2016) 7:3. doi:10.3389/fendo.2016.00003
16. Jaschke H, Neumann S, Moore S, Thomas CJ, Colson AO, Costanzi S, et al. A low molecular weight agonist signals by binding to the transmembrane domain of thyroid-stimulating hormone receptor (TSHR) and luteinizing hormone/chorionic gonadotropin receptor (LHCGR). *J Biol Chem* (2006) 281:9841–4. doi:10.1074/jbc.C600014200
17. Neumann S, Huang W, Titus S, Krause G, Kleinau G, Alberobello AT, et al. Small-molecule agonists for the thyrotropin receptor stimulate thyroid function in human thyrocytes and mice. *Proc Natl Acad Sci U S A* (2009) 106:12471–6. doi:10.1073/pnas.0904506106
18. Latif R, Ali MR, Ma R, David M, Morshed SA, Ohlmeyer M, et al. New small molecule agonists to the thyrotropin receptor. *Thyroid* (2015) 25:51–62. doi:10.1089/thy.2014.0119
19. Neumann S, Nir EA, Eliseeva E, Huang W, Marugan J, Xiao J, et al. A selective TSH receptor antagonist inhibits stimulation of thyroid function in female mice. *Endocrinology* (2014) 155:310–4. doi:10.1210/en.2013-1835
20. Hoyer I, Haas AK, Kreuchwig A, Schulein R, Krause G. Molecular sampling of the allosteric binding pocket of the TSH receptor provides discriminative pharmacophores for antagonist and agonists. *Biochem Soc Trans* (2013) 41:213–7. doi:10.1042/BST20120319
21. Kleinau G, Haas AK, Neumann S, Worth CL, Hoyer I, Furkert J, et al. Signaling-sensitive amino acids surround the allosteric ligand binding site of the thyrotropin receptor. *FASEB J* (2010) 24:2347–54. doi:10.1096/fj.09-149146
22. Cantley AM, Welsch M, Ambesi-Impiombato A, Sanchez-Martin M, Kim MY, Bauer A, et al. Small molecule that reverses dexamethasone resistance in T-cell acute lymphoblastic leukemia (T-ALL). *ACS Med Chem Lett* (2014) 5:754–9. doi:10.1021/ml500044g
23. Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* (2012) 149:1060–72. doi:10.1016/j.cell.2012.03.042
24. Zhang JH, Chung TD, Oldenburg KR, Simple Statistical A. Parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen* (1999) 4:67–73. doi:10.1177/108705719900400206
25. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* (2000) 289:739–45. doi:10.1126/science.289.5480.739
26. Ali MR, Latif R, Davies TF, Mezei M. Monte Carlo loop refinement and virtual screening of the thyroid-stimulating hormone receptor transmembrane domain. *J Biomol Struct Dyn* (2015) 33:1140–52. doi:10.1080/07391102.2014.932310
27. Cui M, Mezei M, Osman R. Prediction of protein loop structures using a local move Monte Carlo approach and a grid-based force field. *Protein Eng Des Sel* (2008) 21:729–35. doi:10.1093/protein/gzn056
28. Mezei M, Zhou MM. Dockres: a computer program that analyzes the output of virtual screening of small molecules. *Source Code Biol Med* (2010) 5:2. doi:10.1186/1751-0473-5-2
29. Leach K, Sexton PM, Christopoulos A. Allosteric GPCR modulators: taking advantage of permissive receptor pharmacology. *Trends Pharmacol Sci* (2007) 28:382–9. doi:10.1016/j.tips.2007.06.004
30. Lipinski CA. Lead- and drug-like compounds: the rule-of-five revolution. *Drug Discov Today Technol* (2004) 1:337–41. doi:10.1016/j.ddtec.2004.11.007
31. Sanders P, Young S, Sanders J, Kabelis K, Baker S, Sullivan A, et al. Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. *J Mol Endocrinol* (2011) 46:81–99. doi:10.1530/JME-10-0127
32. Sanders J, Chirgadze DY, Sanders P, Baker S, Sullivan A, Bhardwaja A, et al. Crystal structure of the TSH receptor in complex with a thyroid-stimulating autoantibody. *Thyroid* (2007) 17:395–410. doi:10.1089/thy.2007.0041
33. Ando T, Latif R, Pritsker A, Moran T, Nagayama Y, Davies TF. A monoclonal thyroid-stimulating antibody. *J Clin Invest* (2002) 110:1667–74. doi:10.1172/JCI0216991
34. Kreuchwig A, Kleinau G, Krause G. Research resource: novel structural insights bridge gaps in glycoprotein hormone receptor analyses. *Mol Endocrinol* (2013) 27:1357–63. doi:10.1210/me.2013-1115
35. Kleinau G, Claus M, Jaeschke H, Mueller S, Neumann S, Paschke R, et al. Contacts between extracellular loop two and transmembrane helix six determine basal activity of the thyroid-stimulating hormone receptor. *J Biol Chem* (2007) 282:518–25. doi:10.1074/jbc.M606176200
36. Galofre JC, Duntas LH, Premawardhana LD. Advances in Graves' disease. *J Thyroid Res* (2012) 2012:809231. doi:10.1155/2012/809231
37. Bahn RS, Burch HB, Cooper DS, Garber JR, Greenlee MC, Klein I, et al. Hyperthyroidism and other causes of thyrotoxicosis: management guidelines of the American Thyroid Association and American Association of Clinical Endocrinologists. *Endocr Pract* (2011) 17:456–520. doi:10.4158/EP.17.3.456
38. Karlsson FA, Axelsson O, Melhus H. Severe embryopathy and exposure to methimazole in early pregnancy. *J Clin Endocrinol Metab* (2002) 87:947–9. doi:10.1210/jc.87.2.947-a
39. Allgeier A, Offermanns S, Van Sande J, Spicher K, Schultz G, Dumont JE. The human thyrotropin receptor activates G-proteins Gs and Gq/11. *J Biol Chem* (1994) 269:13733–5.

40. Boutin A, Eliseeva E, Gershengorn MC, Neumann S. beta-Arrestin-1 mediates thyrotropin-enhanced osteoblast differentiation. *FASEB J* (2014) 28:3446–55. doi:10.1096/fj.14-251124
41. Frenzel R, Voigt C, Paschke R. The human thyrotropin receptor is predominantly internalized by beta-arrestin 2. *Endocrinology* (2006) 147:3114–22. doi:10.1210/en.2005-0687
42. Nagayama Y, Tanaka K, Namba H, Yamashita S, Niwa M. Expression and regulation of G protein-coupled receptor kinase 5 and beta-arrestin-1 in rat thyroid FRTL5 cells. *Thyroid* (1996) 6:627–31. doi:10.1089/thy.1996.6.627
43. Vassart G, Pardo L, Costagliola S. A molecular dissection of the glycoprotein hormone receptors. *Trends Biochem Sci* (2004) 29:119–26. doi:10.1016/j.tibs.2004.01.006
44. van Koppen CJ, de Gooyer ME, Karstens WJ, Plate R, Conti PG, van Achterberg TA, et al. Mechanism of action of a nanomolar potent, allosteric antagonist of the thyroid-stimulating hormone receptor. *Br J Pharmacol* (2012) 165:2314–24. doi:10.1111/j.1476-5381.2011.01709.x
45. Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H, Kobilka BK. Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. *EMBO J* (1997) 16:6737–47. doi:10.1093/emboj/16.22.6737
46. Sheikh SP, Vilardarga JP, Baranski TJ, Lichtarge O, Iiri T, Meng EC, et al. Similar structures and shared switch mechanisms of the beta2-adrenoceptor and the parathyroid hormone receptor. Zn(II) bridges between helices III and VI block activation. *J Biol Chem* (1999) 274:17033–41. doi:10.1074/jbc.274.24.17033
47. Audet M, Bouvier M. Restructuring G-protein-coupled receptor activation. *Cell* (2012) 151:14–23. doi:10.1016/j.cell.2012.09.003
48. Claus M, Jaeschke H, Kleinau G, Neumann S, Krause G, Paschke R. A hydrophobic cluster in the center of the third extracellular loop is important for thyrotropin receptor signaling. *Endocrinology* (2005) 146:5197–203. doi:10.1210/en.2005-0713
49. Schaarschmidt J, Nagel MB, Huth S, Jaeschke H, Moretti R, Hintze V, et al. Rearrangement of the extracellular domain/extracellular loop 1 interface is critical for thyrotropin receptor activation. *J Biol Chem* (2016) 291(27):14095–108. doi:10.1074/jbc.M115.709659
50. Davies TF, Latif R. Targeting the thyroid-stimulating hormone receptor with small molecule ligands and antibodies. *Expert Opin Ther Targets* (2015) 19:835–47. doi:10.1517/14728222.2015.1018181

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 03 October 2017
doi: 10.3389/fendo.2017.00252

Expanding the Role of Thyroid-Stimulating Hormone in Skeletal Physiology

Ramkumarie Baliram[1], Rauf Latif[1], Mone Zaidi[2] and Terry F. Davies[1,2]*

[1] Thyroid Research Unit, Icahn School of Medicine at Mount Sinai and the James J. Peters VA Medical Center, New York, NY, United States, [2] The Mount Sinai Bone Program, Icahn School of Medicine at Mount Sinai, New York, NY, United States

The dogma that thyroid-stimulating hormone (TSH) solely regulates the production of thyroid hormone from the thyroid gland has hampered research on its wider physiological roles. The action of pituitary TSH on the skeleton has now been well described; in particular, its action on osteoblasts and osteoclasts. It has also been recently discovered that the bone marrow microenvironment acts as an endocrine circuit with bone marrow-resident macrophages capable of producing a novel TSH-β subunit variant (TSH-βv), which may modulate skeletal physiology. Interestingly, the production of this TSH-βv is positively regulated by T3 accentuating such modulation in the presence of thyroid overactivity. Furthermore, a number of small molecule ligands acting as TSH agonists, which allosterically modulate the TSH receptor have been identified and may have similar modulatory influences on bone cells suggesting therapeutic potential. This review summarizes our current understanding of the role of TSH, TSH-β, TSH-βv, and small molecule agonists in bone physiology.

Keywords: TSH-β, TSH-βv, TSH-receptor, macrophage, osteoblast, osteoclast

OPEN ACCESS

Edited by:
Alessandro Antonelli, University of Pisa, Italy

Reviewed by:
Marco Centanni, Sapienza Università di Roma, Italy
Ilaria Ruffilli, University of Pisa, Italy

****Correspondence:***
Ramkumarie Baliram
annie.baliram@mssm.edu

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *28 June 2017*
Accepted: *13 September 2017*
Published: *03 October 2017*

Citation:
Baliram R, Latif R, Zaidi M and Davies TF (2017) Expanding the Role of Thyroid-Stimulating Hormone in Skeletal Physiology. Front. Endocrinol. 8:252. doi: 10.3389/fendo.2017.00252

INTRODUCTION

The skeleton has a wide range of functions, which include structural support/protection, locomotion, and mineral homeostasis. In addition, the emerging role of bone as an endocrine unit is rapidly gaining momentum because bone secretes a variety of hormones such as osteocalcin, osteoprotegerin, osteoclastogenesis inhibitory factor, sclerostin, and fibroblast growth factor 23 (1), and it has recently also been shown to be the source of a variant form of TSH-β subunit (2–4). Bone is derived from intramembranous ossification of fibrous membranes and from endochondrial ossification of hyaline cartilage during fetal development. Osteoblasts and osteoclasts, two major cell types found in bone, are derived from unique cell lineages. Osteoblasts differentiate from the mesenchymal lineage while osteoclasts are from the hematopoietic stem cell lineage. The close balance in their activity during bone remodeling between the osteoblasts-inducing bone deposition and osteoclasts-inducing bone resorption appears to be crucial for precise maturation and preservation of bone integrity. However, the bony skeleton can be structurally and functionally altered by various diseases, drugs and extra-skeletal hormones, growth factors, and cytokines as well as mechanical forces (1).

An overactive thyroid gland has long been known to be associated with significant bone loss (5). Osteoporosis is seen in many overt hyperthyroid states, most commonly Graves' disease and toxic multinodular goiter (6–9). In addition, excessive thyroid hormone replacement therapy in

postmenopausal women is known to contribute to bone loss (10). Hence, bone turnover is increased and bone mass decreased when thyroid hormone levels are high and TSH levels are low and such changes in bone can also be seen in animal models (5, 11). In these conditions under which bone is loss, TSH levels in the serum fall to insignificant concentrations, but thyroid hormones (T3 and T4) may vary from high to normal, thus arguing for a role of TSH or other TSH receptor agonists in preventing bone loss. This review highlights the role of TSH, TSH-βv, and small molecules on skeletal biology.

THE HYPOTHALAMIC–PITUITARY AXIS

Thyrotropin-releasing hormone (TRH) also known as thyroliberin was first isolated by Schally and Guillemin in 1969 (12, 13). TRH is synthesized in the paraventricular nucleus of the hypothalamus and it regulates both the synthesis and release of TSH from the anterior pituitary (14). The production of the TSH-β subunit in the pituitary is regulated by both the CREB-binding transcription factors and the pituitary-specific transcription factor-1 (14) while thyroid hormone levels (T4 and T3) being preserved by a negative feedback loop (**Figure 1**). TSH in turn acts through the thyroid-stimulating hormone receptor (TSHR) to induce the synthesis and release of T4 and a smaller amount of T3 with additional T3 being derived by peripheral deiodination (15). Additionally, thyroid hormones then exert actions through the thyroid hormone receptors (TRs) to inhibit TRH and TSH synthesis and its secretion. As such, when thyroid hormones are high, the TSH levels are low.

STRUCTURE AND FUNCTION OF THE TSH MOLECULE

Thyroid-stimulating hormone (TSH) and follicle-stimulating hormone, along with chorionic gonadotropin (hCG) and luteinizing hormone, are heterodimeric proteins that share a common α-chain and unique β-chains, which confer hormone specificity. Both mouse and man TSH-β subunits share significant homology (16, 17). In these species, the TSH-β contains 138 amino acids with 20 of them representing the signal peptide and the other 118 the mature protein. The common α-chain is made up of 92 amino acids. The α-subunit gene shows a general expression pattern compared to the TSH-β subunit gene expression, which is restricted to the anterior pituitary. Although TSH-α and TSH-β are transcribed from different genes, it is generally understood that the molecular interaction of the α-subunit and the TSH-β subunit confers specificity to the molecule (18). TSH interacts with the G-protein-coupled TSHR (19, 20) in controlling thyroid function and it also has extrathyroidal activity *via* TSHR expression at a variety of sites (21). Of relevance here is that pituitary TSH has been shown to be osteoprotective *in vitro* and *in vivo* by activating osteoblasts and inhibiting osteoclasts and this will be reviewed further.

Mouse studies have clearly shown that there is *in vivo* osteoprotective activity associated with the TSHR itself even when pituitary TSH is suppressed by excessive thyroid hormone (11). These data indicate that either the intrinsic, constitutive, activity of the TSHR itself is able to provide the protection in the absence of TSH ligand or raised the possibility of a local TSHR stimulator being available to maintain TSHR signaling in the absence of pituitary TSH. This possibility prompted us to search for other isoform (s) of the TSH molecule in bone.

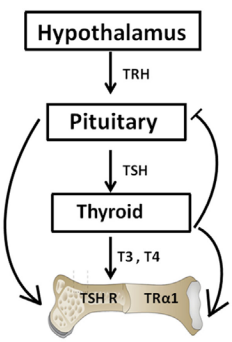


FIGURE 1 | The hypothalamic–pituitary–thyroid–bone axis. This simplified figure illustrates the interactions of the pituitary hormone thyroid-stimulating hormone (TSH) and thyroid hormones T3 and T4 with bone. A negative feedback loop with origins in the thyroid and projections to the pituitary and hypothalamus is depicted. T3 and T4 hormone levels are maintained by such a loop. The role of TSH on bone has been hampered by the dogma that TSH exerts functions exclusively within the thyroid. However, within the past few decades, TSH has been show to exert physiologic effects on both osteoblasts and osteoclasts.

A NOVEL TSH-β SUBUNIT VARIANT IN PITUITARY AND BONE MARROW

In fact, extrapituitary sources of TSH have long been known (22, 23). Hence, parallel to the pituitary-thyroid endocrine circuit, there are additional TSH-related circuits that function beyond the thyroid and involves the immune system as evidenced by reports, which shows that immune cells are capable of producing TSH (22) and a novel TSH-βv is produced within the bone marrow cells; primarily by macrophages (2–4).

In the mouse (**Figure 2A**), unlike the human (**Figure 2B**), the TSH-β coding region is located in segments of exons 4 and 5. In the novel mouse, TSH-β splice variant (TSH-βv) exon 4 is missing. The human TSH gene contains three exonic sequences but exon-2 is missing in the hTSH-βv. Molecular docking and experimental studies suggested that TSH-β and TSH-βv were able to bind and signal through the TSHR (2, 3). Further, molecular docking studies have also shown that the binding affinity of TSH-βv is comparable to the native TSH-β subunit (2). Of direct relevance here is that it has been shown that the mouse pituitary in addition to macrophages is also a source of this novel TSH-β splice variant (TSH-βv), which may retain its biological effect (2–4).

In the human, TSH-β is similarly expressed primarily in the thyrotrophs of the anterior pituitary gland. But we and others have also observed, as in the mouse, that a TSH-βv is expressed in human pituitary, human bone marrow, and in human peripheral blood-derived macrophages (3, 24). These data further support the concept of an extrapituitary TSH-like molecule, which can

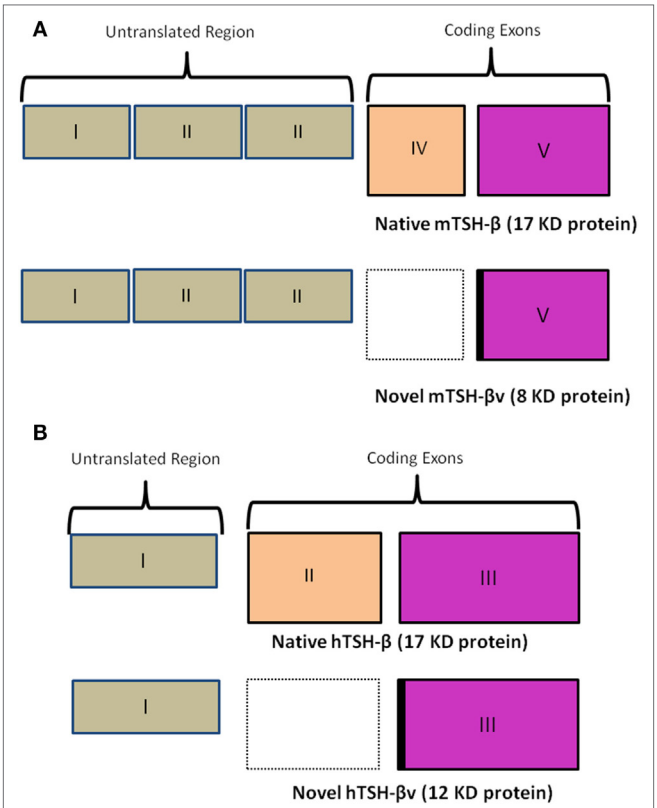


FIGURE 2 | **(A)** A schematic comparison of the mouse native TSH-β and novel TSH-βv. Of note is a missing exon IV in the splice variant resulting in a smaller peptide of 8 vs 17 kDa for the full length. The intronic region is marked in black. Copyright (2013) Endocrinology and reproduced with permission from Oxford University Press (2). **(B)** A similar schematic outlining the human native TSH-β and novel TSH-βv gene arrangement [adapted from Baliram et al. (3)]. Copyright (2013) Endocrinology and reproduced with permission from Oxford University Press (2).

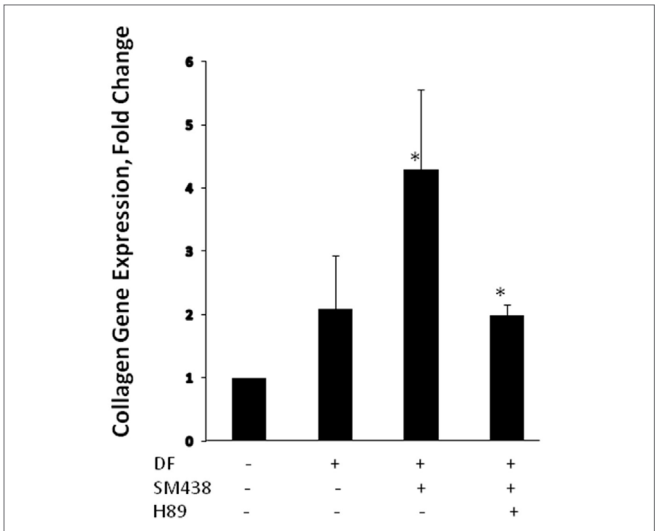


FIGURE 3 | Small molecule MS 438 enhanced collagen gene expression in human osteoblast cells. The hFOB 1.19 cells were transformed into osteoblasts by treating with osteogenic stimulation/differentiation factors—20 μm of beta-glycerophosphate, 50 μg/ml of ascorbic acid, and 10^{-7}M dexamethasone in refreshed media every 3 days along with or without 10 μM of MS 438 and 10 μM of PKA inhibitor (H 89). On day 10, cultures were terminated and gene expression analyzed by quantitative PCR and showed enhanced collagen gene expression.

bind to TSHRs on osteoblasts and osteoclasts to initiate proliferation and differentiation. However, the full significance of this conclusion in bone biology needs to be further elucidated.

TSHR AND SMALL MOLECULE AGONISTS

In recent years, small molecules have gained momentum as therapeutic options for modulating TSHR signaling (25). In addition to their low cost of manufacturing, these molecules have the biological advantage of easily crossing the plasma membrane and binding to allosteric sites on the receptor. Their chemical nature renders them resistant to proteolytic enzymes and thus ideal therapeutic agents. A few potent small molecule agonists to the TSHR have been reported (26–28). These molecules interact with the TSHR on distinct polar and non-polar residues within the hydrophobic pockets created by the helices of the receptor transmembrane domains, thereby exerting a stimulatory effect by altering the interaction and movement of these helices (29, 30). Our laboratory has reported a small molecule (MS-438) (28), which appears to increase osteoblast formation through the PKA signaling pathway (**Figure 3**). Other studies have also shown biological action of small molecules on bone cells overexpressing the TSHR (31) and two small molecule TSHR antagonists have been reported but with lower affinity than likely to be clinically useful (27, 32).

TSHR GENE EXPRESSION

The human TSHR gene, cloned in 1989 (19, 20), is on chromosome #14q-31 and codes for a seven transmembrane, G-protein-coupled receptor. The TSHR is the largest of the glycoprotein receptor because of its 8- and 50 amino acid insertions into the ectodomain (residues 38–45 and 317–367). The TSHR is described as a G protein-coupled receptor with both $G_{\alpha s}$ and $G_{\alpha q}$ as primary effectors and with constitutive activity, which is enhanced further by TSH or by stimulating TSHR autoantibodies (33). The minimal 5′ promoter is necessary to confer thyroid-specific expression and cAMP autoregulation (34). It is well established that in addition to the $G_{\alpha s}$ cAMP/protein kinase A/ERK signaling cascade, TSH activates $G_{\alpha q}$-AKT/protein kinase C/Ca 2+ coupled signaling networks predominantly at high concentrations (35). Although the TSHR is important for growth and function of the thyroid gland, as discussed earlier, it has a diverse expression profile including on lymphocytes, macrophages, adipose tissue, fibroblasts, heart, and bone among others (21).

TSH EFFECTS ON OSTEOBLASTS

Osteoblast-related cells such as bone lining cells, stromal cells, preosteoblasts osteoprogenitors, osteoblasts, and osteocytes are differentiated from mesenchymal cells. These cells can also

terminally differentiate into fibroblasts, chondrocytes, myoblasts, and adipocytes (36). Osteoblast lineage cells perform varied functions, which include support for muscle attachment and lend itself as a reservoir for minerals such as phosphorus and calcium. Additionally, osteocytes derived from osteoblast lineage cells produce FGF 23 (37). Osteoblast lineage cells contribute to the bone marrow niche (38) and are also involved in insulin action (39, 40).

Osteoblast formation requires a series of sequential steps starting from precursor cell commitment, then cell proliferation and then cell differentiation, which is marked by type-1 collagen formation and matrix deposition. Once bone is formed, osteoblasts then go onto differentiate into osteocytes (41). Expression of the TSHR in the rat osteoblast line UMR106 cells was first demonstrated (42). Then in subsequent studies, the TSHR mRNA expression and protein were observed in normal osteoblasts (43–49).

Thyroid-stimulating hormone was found to induce genes involved in the regulation and differentiation of mesenchymal stem cells within the bone marrow (50) and treatment of osteoblasts with TSH *in vitro* has been shown in most studies to have stimulatory effects on osteoblast differentiation and function (31, 46, 47). Inhibition of low-density lipoprotein receptor-related protein 5 mRNA by TSH suggested a role for TSH on osteoblastogenesis. TSH has since been shown to activate Wnt-5a signaling in osteoblast differentiation (47). Similarly, in embryonic stem cell cultures, TSH-stimulated osteoblast differentiation *via* protein kinase C and the non-canonical Wnt-5a pathway (47). Further, TSH also stimulated proliferation and differentiation, as shown by an upregulation in alkaline phosphatase and in increase in IGF-1 and IGF-2 mRNA expressions (51). Recently, TSH was shown to stimulate arrestin 1, which leads to the activation of intracellular signaling molecules such as ERK, P38 MAPK, and AKT (31).

TSH EFFECTS ON OSTEOCLASTS

Osteoclasts are terminally differentiated polykaryons, which reabsorb bone matrix and mineral. They attach to bone through αVβ3 integrin that interacts with bone matrix proteins. These interactions form cytoplasmic extensions with finger-like processes known as the ruffled border. These borders function to increase the surface area when contacting bone and through them, osteoclasts secrete hydrochloric acid from acidic vacuoles. The acid dissolves bone mineral and also activates acid hydrolases, such as cathepsin K in degrading the matrix (52, 53).

Osteoclasts differentiate through the commitment of hematopoietic stem cells to the myeloid lineage and are regulated by PU.1 together with micro-ophthalmia-associated transcription factors (54). Also, macrophage CSF/CSF-1R stimulates expression of RANK and leads to osteoclast precursor commitment. Furthermore, RANK Ligand (RANKL) is essential for osteoclast formation, function, and survival (52). Moreover, RANKL/RANK signaling induces the nuclear factor-κB (NF-κB) and nuclear factor of activated T cells cytoplasmic 1, which leads to osteoclast differentiation (55).

Recent studies have demonstrated that TSH reduces osteoclastogenesis by acting on their TSHR G-protein-coupled receptor (43, 56, 57). In animal studies, mice which lack the TSHR exhibited osteoporosis because of enhanced osteoclast formation (43). TNFα, which is a member of the tumor necrosis family, is a well-established signal that increases osteoclasts (58). The receptor activator for NFκB ligand (RANKL) stimulates endogenous TNFα expression and it is necessary for osteoclast formation. Additionally, RANKL and a mixture with IL1 and TNFα increase osteoclastogenesis (59). Moreover, we showed that the TSHR null mice exhibit an elevated TNFα expression in osteoclast progenitors. The fact that these mice develop osteoporosis (43) suggests that TNFα overproduction may play a major role in the development of this condition since TSH has been shown to directly downregulate TNFα transcription induced by IL1 or RANKL treatments (59).

TSH EFFECTS ON OSTEOCYTES

In contrast to osteoblasts and osteoclasts, osteocytes make up 90–95% of bone cells and are embedded in bone matrix for decades. Osteocytes have been increasingly recognized as the major orchestrator of bone activity, particularly considering the fact that they secrete a 190-amino-acid glycoprotein, which decreases bone formation by inhibiting terminal osteoblast differentiation while promoting apoptosis. These cells also regulate osteoblast physiology by controlling osteoblast and osteoclast activity during bone remodeling. Terminally differentiated osteoblasts are widely described as mature osteocytes.

However, it is poorly understood how osteoblast becomes embedded in bone matrix to begin a new life in the capacity as an osteocyte and also the molecular and genetic mechanisms, which regulate the differentiation and maturation of the osteocyte are also poorly understood (60).

Osteocytes takes up residence in lacunae within mineralized matrix and protrude their dendritic processes through the canaliculi to form a network, which connects with cells on the bone surface and to blood vessels (61).

Localized conditions such as mechanical stresses and microdamage stimulate osteocytes to release cytokines, chemotactic signals, or to induce apoptosis. An, increase in mechanical stress stimulates local bone formation through osteoblast activity, whereas reduced microdamage results in bone resorption induced by osteoclast activity (60, 62, 63). These mechanosensor capabilities of osteocytes allow them to control bone remodeling through their regulation of osteoclasts and osteoblasts *via* the RANKL/RANK pathway and modulation of Wnt signaling (60, 64). The effects of TSH on osteocytes have not been studied.

SKELETAL CONSEQUENCES IN THE TSHR KNOCKOUT MOUSE

The use of animal models in the study of TSH effects on bone has provided important fundamental advances. Animal models of hypothyroid mice such as the Snell Dwarf mouse (65), the cog mouse (66), and the hyt/hyt mouse (67, 68) have all retained the TSHR expression and ligand-independent constitutive signals transmitted by the TSHR (69). In contrast, the generation of the TSHR-KO mouse, brought a novel way of studying TSH signaling

and this implicated the TSHR in bone biology (11, 43, 70). In this mouse, exon-1 of the TSHR gene was replaced with a green fluorescent protein (GFP) cassette. The heterozygotes, haploinsufficient in the TSHR, were euthyroid and exhibit normal growth and normal thyroid hormone and TSH levels. By contrast, the homozygotes (TSHR-KO mice) showed runted growth, low thyroid hormone levels, and very high TSH levels and required thyroid hormone replacement for normal growth and survival. Nevertheless, these mice had a smaller thyroid gland in the correct position. An examination of the TSHR-KO thyroid follicles (**Figure 4**) showed GFP expression in the heterozygote and homozygote thyroid follicles indicating that the TSHR had been deleted but the thyroid follicles, while appearing normal in the heterozygous, were few and small in the homozygous and their pattern was disorganized. Hence, the TSHR-KO mice showed congenital hypothyroidism with undetectable thyroid hormones and a rise in serum TSH.

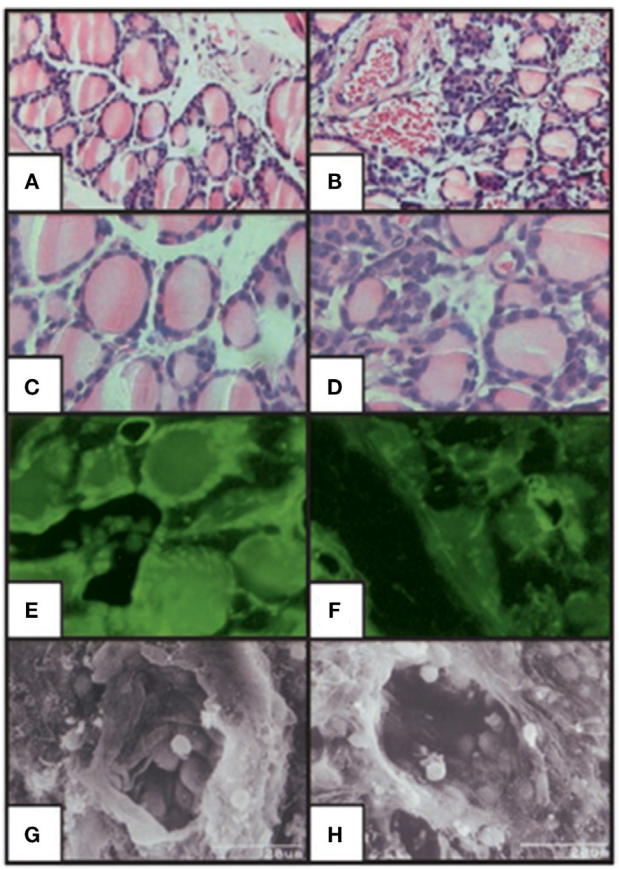


FIGURE 4 | Histologic staining of the thyroid-stimulating hormone receptor (TSHR)-KO mouse thyroid gland. Hematoxylin/eosin stained sections of WT **(A,C)** and TSHR-KO **(B,D)** thyroids. Fluorescent imaging of heterozygous **(E)** and TSHR-KO **(F)** green fluorescent protein reporter gene expression in the thyroid. Scanning electron micrographs for WT **(G)** and TSHR-KO **(H)** thyroid follicles [magnification: **(A,B)** 100; **(C–F)** 400; **(G,H)** 1,500]. Note the small but present thyroid cells in the TSHR-KO mouse, which has been used in a number of studies elucidating thyroid-stimulating hormone actions on bone. Copyright (2002) National Academy of Sciences, U.S.A (71).

Untreated TSHR-KO mice were found to have a low bone mineral density (BMD), increase bone formation, and resorption. However, even when these mice were given thyroid hormone replacement, they displayed a reduction in BMD and reduced calvarial thickness (43). Heterozygotes showed a smaller reduction in BMD, affecting only some parts of the skeleton. There were no change in calvarial thickness, and no difference in bone resorption or formation. These data indicated that TSH signaling must suppress bone loss and TSH was, therefore, proposed as an activator of bone formation and inhibitor of bone resorption (43). Because the TSHR-KO mice are only thyroid supplemented from weaning (around 21 days of age) (43), they do remain severely hypothyroid during a critical time of skeletal development but clearly are unable to catch up.

TSH EFFECTS ON NORMAL RODENT SKELETON

The osteoporosis due to TSHR deficiency in the TSHR/KO mouse is of the high-turnover variety. Further, when TSH was intermittently administered into ovariectomized rats, it displayed a robust *in vivo* antiresorptive action (46, 72). TSH increased bone volume, trabecular number, trabecular thickness, and decreased trabecular separation (46). TSH also decreased osteoclast numbers in these rats (46) suggesting that TSH treatment is capable of restoring ovariectomy-induced bone loss and bone strength (72). The inhibitory action of TSH on osteoclast even persisted after therapy halted (72). This lasting antiresorptive action of TSH was mimicked in cells that genetically overexpressed a constitutively active ligand-independent TSHR (73). Additionally, due to a loss of function in congenital mutant TSHR congenital hypothyroid mice, osteoclast differentiation is activated, thus confirming that TSHR signaling has a pivotal role in the regulation of bone remodeling (72).

TSH EFFECTS ON THE HUMAN SKELETON

As discussed earlier, new lines of evidence have shown the influence of pituitary hormones on the skeleton (43, 72, 74, 75). For example, suppressed hyperthyroid levels of TSH are well known to correlate with low BMD (76), especially in postmenopausal women, and even low normal TSH levels show the same relationship in the elderly (77) and an increased risk of hip fractures in euthyroid women (77). These studies also show that duration of TSH suppression was also a predictor of major osteoporotic fractures. However, others (78) could not distinguish the separate pharmacological effects of thyroid hormones and TSH on bone turnover, although TSH was correlated inversely with markers indicative of bone turnover and is unrelated to thyroid hormones.

For humans, few large data sets exist on the physiologic effects of TSH on bone in. However, recombinant human TSH administration regulated C-telopeptides type-1 collagen levels and alkaline phosphatase with no effect on levels of osteoprotegerin (79) or on the receptor activator of nuclear factor-κB ligand levels (80).

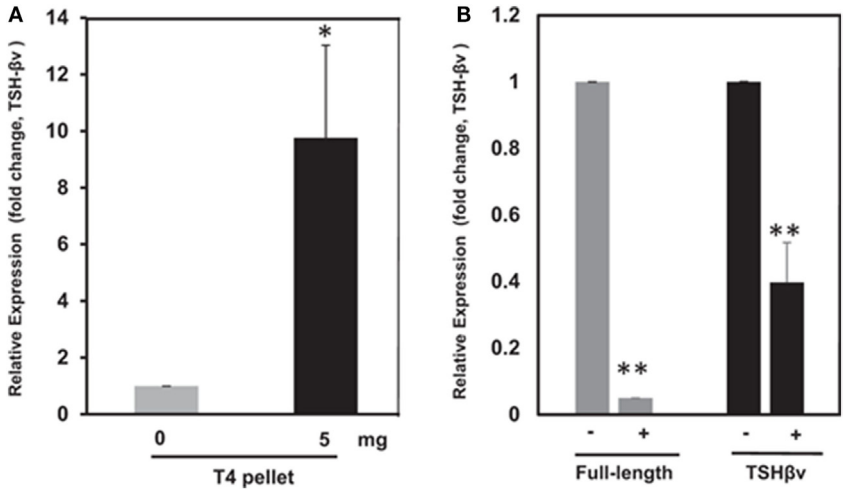


FIGURE 5 | **(A)** Thyroid hormone regulation of mouse thyroid-stimulating hormone (TSH)-βv in bone marrow cells. Here, bone marrow cells from mouse WT mice subcutaneously treated with T4 hormone pellets for 21 days showed greatly increased TSH-βv gene expression. Copyright (2016) Endocrinology and reproduced with permission from Oxford University Press (3). **(B)** Thyroid hormone regulation of mouse TSH-βv in the pituitary. The pituitary tissue from WT mice administered subcutaneous T4 pellets for 21 days showed suppression of both wild-type TSH-β and TSH-βv. This is in contrast to the bone marrow cells shown in **(B)**. Copyright (2016) Endocrinology and reproduced with permission from Oxford University Press (3).

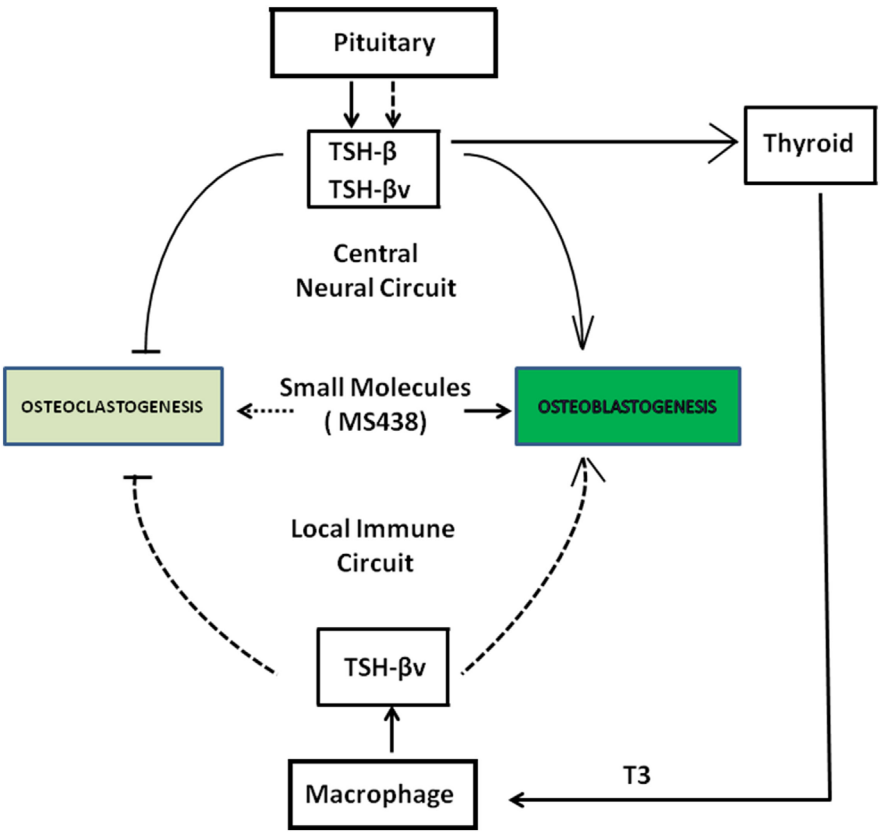


FIGURE 6 | Schematic showing a collaborative effort among pituitary, thyroid, and macrophages in the local bone marrow microenvironment in regulating osteoclast and osteoblast activity through the release of thyroid-stimulating hormone (TSH)-β and TSH-βv. SM 438 is a small molecule agonist at the thyroid-stimulating hormone receptor. Solid dark arrows indicate modulatory effects of TSH-β, TSH-βv, and SM 438 on bone cells and the broken arrows indicate that such effects still need to be mapped.

T3 EFFECTS ON THE SKELETON

Thyroid hormone levels have a major influence on bone homeostasis (81), and this has been well reviewed elsewhere (5). Investigators have focused on the direct effects of the active thyroid hormone (T3), on bone cells, *via* the thyroid hormone receptor family that induces transcription in a ligand-dependent manner (82). Osteoblasts express thyroid hormone receptors (TRs) (TRα1, TRα2, and TRβ1) and respond to T3 with increased proliferation and expression of lineage-specific markers such as alkaline phosphatase, osteocalcin, and collagen. Interestingly, although osteoclasts have TRs, their response to T3 appears to be mediated mostly by osteoblasts since T3 induces osteoblasts to express RANKL, the key osteoclastogenic cytokine. Additionally, mice lacking the known active isoforms of TRs have retarded bone growth and maturation, but do not manifest increased BMD, as would be predicted if T3 was an important stimulus of bone resorption in the euthyroid state (83). Further, T4, the prohormone of T3, suppressed pituitary TSH release but enhanced bone marrow TSHβv expression (3) (**Figures 5A,B**) indicating an attempt at osteoprotection. Hence, our observation of enhanced bone loss induced by T4 when the TSHR is absent fits with these correlative data (11).

SUMMARY AND CONCLUSION

Thyroid-stimulating hormone, TSH-β, and TSH-βv are produced through central neural circuits in the pituitary thyrotrophs and are negatively regulated by T3 produced by the thyroid gland. However, in the local peripheral immune circuit, only TSH-βv is produced by bone marrow macrophages and appears to be positively regulated by T3. It has been shown that intact TSH exerts anabolic and osteoprotective effects on bone by stimulating osteoblast differentiation and by inhibiting osteoclast formation and survival. Since the TSHR is widely distributed in bone cells, the production of TSH-βv by macrophages argues for a local TSH-TSHR circuit regulating bone physiology. Evidence for the importance of such influences is shown by the greater T4-induced bone loss in the absence of TSHR signaling (**Figure 6**).

AUTHOR CONTRIBUTIONS

RB, RL, MZ, and TF contributed to the design, figures, and writing of this manuscript.

ACKNOWLEDGMENTS

The authors are grateful for the support of Drs. Risheng Ma and Syed Morshed in the Davies Laboratory and Drs. Sun Li and Tony Yuen in the Zaidi laboratory.

FUNDING

This study was supported by NIH grants: DK069713 and Veterans Affairs Merit Award.

REFERENCES

1. Guntur AR, Rosen CJ. Bone as an endocrine organ. *Endocr Pract* (2012) 18:758–62. doi:10.4158/EP12141.RA
2. Baliram R, Chow A, Huber AK, Collier L, Ali MR, Morshed SA, et al. Thyroid and bone: macrophage-derived TSH-beta splice variant increases murine osteoblastogenesis. *Endocrinology* (2013) 154:4919–26. doi:10.1210/en.2012-2234
3. Baliram R, Latif R, Morshed SA, Zaidi M, Davies TF. T3 regulates a human macrophage-derived TSH-beta splice variant: implications for human bone biology. *Endocrinology* (2016) 157:3658–67. doi:10.1210/en.2015-1974
4. Vincent BH, Montufar-Solis D, Teng BB, Amendt BA, Schaefer J, Klein JR. Bone marrow cells produce a novel TSHbeta splice variant that is upregulated in the thyroid following systemic virus infection. *Genes Immun* (2009) 10:18–26. doi:10.1038/gene.2008.69
5. Bassett JH, Williams GR. Role of thyroid hormones in skeletal development and bone maintenance. *Endocr Rev* (2016) 37:135–87. doi:10.1210/er.2015-1106
6. De Menis E, Da Rin G, Roiter I, Legovini P, Foscolo G, Conte N. Bone turnover in overt and subclinical hyperthyroidism due to autonomous thyroid adenoma. *Horm Res* (1992) 37:217–20. doi:10.1159/000182315
7. Kisakol G, Kaya A, Gonen S, Tunc R. Bone and calcium metabolism in subclinical autoimmune hyperthyroidism and hypothyroidism. *Endocr J* (2003) 50:657–61. doi:10.1507/endocrj.50.657
8. Foldes J, Tarjan G, Szathmari M, Varga F, Krasznai I, Horvath C. Bone mineral density in patients with endogenous subclinical hyperthyroidism: is this thyroid status a risk factor for osteoporosis? *Clin Endocrinol (Oxf)* (1993) 39:521–7. doi:10.1111/j.1365-2265.1993.tb02403.x
9. Gurlek A, Gedik O. Effect of endogenous subclinical hyperthyroidism on bone metabolism and bone mineral density in premenopausal women. *Thyroid* (1999) 9:539–43. doi:10.1089/thy.1999.9.539
10. Vestergaard P, Rejnmark L, Weeke J, Mosekilde L. Fracture risk in patients treated for hyperthyroidism. *Thyroid* (2000) 10:341–8. doi:10.1089/thy.2000.10.341
11. Baliram R, Sun L, Cao J, Li J, Latif R, Huber AK, et al. Hyperthyroid-associated osteoporosis is exacerbated by the loss of TSH signaling. *J Clin Invest* (2012) 122:3737–41. doi:10.1172/JCI63948
12. Boler J, Enzmann F, Folkers K, Bowers CY, Schally AV. The identity of chemical and hormonal properties of the thyrotropin releasing hormone and pyroglutamyl-histidyl-proline amide. *Biochem Biophys Res Commun* (1969) 37:705–10. doi:10.1016/0006-291X(69)90868-7
13. Burgus R, Dunn TF, Desiderio D, Guillemin R. [Molecular structure of the hypothalamic hypophysiotropic TRF factor of ovine origin: mass spectrometry demonstration of the PCA-His-Pro-NH2 sequence]. *C R Acad Sci Hebd Seances Acad Sci D* (1969) 269:1870–3.
14. Hashimoto K, Zanger K, Hollenberg AN, Cohen LE, Radovick S, Wondisford FE. cAMP response element-binding protein-binding protein mediates thyrotropin-releasing hormone signaling on thyrotropin subunit genes. *J Biol Chem* (2000) 275:33365–72. doi:10.1074/jbc.M006819200
15. Bianco AC, Kim BW. Deiodinases: implications of the local control of thyroid hormone action. *J Clin Invest* (2006) 116:2571–9. doi:10.1172/JCI29812
16. Szkudlinski MW, Fremont V, Ronin C, Weintraub BD. Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. *Physiol Rev* (2002) 82:473–502. doi:10.1152/physrev.00031.2001
17. Gordon DF, Wood WM, Ridgway EC. Organization and nucleotide sequence of the gene encoding the beta-subunit of murine thyrotropin. *DNA* (1988) 7:17–26. doi:10.1089/dna.1988.7.17
18. Shupnik MA, Ridgway EC, Chin WW. Molecular biology of thyrotropin. *Endocr Rev* (1989) 10:459–75. doi:10.1210/edrv-10-4-459
19. Vassart G, Dumont JE. The thyrotropin receptor and the regulation of thyrocyte function and growth. *Endocr Rev* (1992) 13:596–611. doi:10.1210/edrv-13-3-596
20. Nagayama Y, Kaufman KD, Seto P, Rapoport B. Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. *Biochem Biophys Res Commun* (1989) 165:1184–90. doi:10.1016/0006-291X(89)92727-7

21. Davies T, Marians R, Latif R. The TSH receptor reveals itself. *J Clin Invest* (2002) 110:161–4. doi:10.1172/JCI0216234
22. Blalock JE. The immune system as the sixth sense. *J Intern Med* (2005) 257:126–38. doi:10.1111/j.1365-2796.2004.01441.x
23. Smith EM, Phan M, Kruger TE, Coppenhaver DH, Blalock JE. Human lymphocyte production of immunoreactive thyrotropin. *Proc Natl Acad Sci U S A* (1983) 80:6010–3. doi:10.1073/pnas.80.19.6010
24. Schaefer JS, Klein JR. A novel thyroid stimulating hormone beta-subunit isoform in human pituitary, peripheral blood leukocytes, and thyroid. *Gen Comp Endocrinol* (2009) 162:241–4. doi:10.1016/j.ygcen.2009.04.006
25. Davies TF, Latif R. Targeting the thyroid-stimulating hormone receptor with small molecule ligands and antibodies. *Expert Opin Ther Targets* (2015) 19:835–47. doi:10.1517/14728222.2015.1018181
26. Jaschke H, Neumann S, Moore S, Thomas CJ, Colson AO, Costanzi S, et al. A low molecular weight agonist signals by binding to the transmembrane domain of thyroid-stimulating hormone receptor (TSHR) and luteinizing hormone/chorionic gonadotropin receptor (LHCGR). *J Biol Chem* (2006) 281:9841–4. doi:10.1074/jbc.C600014200
27. Neumann S, Huang W, Titus S, Krause G, Kleinau G, Alberobello AT, et al. Small-molecule agonists for the thyrotropin receptor stimulate thyroid function in human thyrocytes and mice. *Proc Natl Acad Sci U S A* (2009) 106:12471–6. doi:10.1073/pnas.0904506106
28. Latif R, Ali MR, Ma R, David M, Morshed SA, Ohlmeyer M, et al. New small molecule agonists to the thyrotropin receptor. *Thyroid* (2015) 25:51–62. doi:10.1089/thy.2014.0119
29. Kleinau G, Haas AK, Neumann S, Worth CL, Hoyer I, Furkert J, et al. Signaling-sensitive amino acids surround the allosteric ligand binding site of the thyrotropin receptor. *FASEB J* (2010) 24:2347–54. doi:10.1096/fj.09-149146
30. Cantley AM, Welsch M, Ambesi-Impiombato A, Sanchez-Martin M, Kim MY, Bauer A, et al. Small molecule that reverses dexamethasone resistance in T-cell acute lymphoblastic leukemia (T-ALL). *ACS Med Chem Lett* (2014) 5:754–9. doi:10.1021/ml500044g
31. Boutin A, Eliseeva E, Gershengorn MC, Neumann S. beta-Arrestin-1 mediates thyrotropin-enhanced osteoblast differentiation. *FASEB J* (2014) 28:3446–55. doi:10.1096/fj.14-251124
32. Latif R, Realubit RB, Karan C, Mezei M, Davies TF. TSH receptor signaling abrogation by a novel small molecule. *Front Endocrinol* (2016) 7:130. doi:10.3389/fendo.2016.00130
33. Latif R, Morshed SA, Zaidi M, Davies TF. The thyroid-stimulating hormone receptor: impact of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on multimerization, cleavage, and signaling. *Endocrinol Metab Clin North Am* (2009) 38:319–41, viii. doi:10.1016/j.ecl.2009.01.006
34. Ikuyama S, Niller HH, Shimura H, Akamizu T, Kohn LD. Characterization of the 5'-flanking region of the rat thyrotropin receptor gene. *Mol Endocrinol* (1992) 6:793–804. doi:10.1210/mend.6.5.1318504
35. Morshed SA, Latif R, Davies TF. Characterization of thyrotropin receptor antibody-induced signaling cascades. *Endocrinology* (2009) 150:519–29. doi:10.1210/en.2008-0878
36. Matsuo K, Irie N. Osteoclast-osteoblast communication. *Arch Biochem Biophys* (2008) 473:201–9. doi:10.1016/j.abb.2008.03.027
37. Razzaque MS. The FGF23-Klotho axis: endocrine regulation of phosphate homeostasis. *Nat Rev Endocrinol* (2009) 5:611–9. doi:10.1038/nrendo.2009.196
38. Long F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* (2011) 13:27–38. doi:10.1038/nrm3254
39. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, et al. Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. *Cell* (2010) 142:296–308. doi:10.1016/j.cell.2010.06.003
40. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. *Cell* (2010) 142:309–19. doi:10.1016/j.cell.2010.06.002
41. Martin TJ, Sims NA, Ng KW. Regulatory pathways revealing new approaches to the development of anabolic drugs for osteoporosis. *Osteoporos Int* (2008) 19:1125–38. doi:10.1007/s00198-008-0575-5
42. Inoue M, Tawata M, Yokomori N, Endo T, Onaya T. Expression of thyrotropin receptor on clonal osteoblast-like rat osteosarcoma cells. *Thyroid* (1998) 8:1059–64. doi:10.1089/thy.1998.8.1059
43. Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y, et al. TSH is a negative regulator of skeletal remodeling. *Cell* (2003) 115:151–62. doi:10.1016/S0092-8674(03)00771-2
44. Bassett JH, Williams AJ, Murphy E, Boyde A, Howell PG, Swinhoe R, et al. A lack of thyroid hormones rather than excess thyrotropin causes abnormal skeletal development in hypothyroidism. *Mol Endocrinol* (2008) 22:501–12. doi:10.1210/me.2007-0221
45. Bassett JH, van der Spek A, Logan JG, Gogakos A, Bagchi-Chakraborty J, Murphy E, et al. Thyrostimulin regulates osteoblastic bone formation during early skeletal development. *Endocrinology* (2015) 156:3098–113. doi:10.1210/en.2014-1943
46. Sampath TK, Simic P, Sendak R, Draca N, Bowe AE, O'Brien S, et al. Thyroid-stimulating hormone restores bone volume, microarchitecture, and strength in aged ovariectomized rats. *J Bone Miner Res* (2007) 22:849–59. doi:10.1359/jbmr.070302
47. Baliram R, Latif R, Berkowitz J, Frid S, Colaianni G, Sun L, et al. Thyroid-stimulating hormone induces a Wnt-dependent, feed-forward loop for osteoblastogenesis in embryonic stem cell cultures. *Proc Natl Acad Sci U S A* (2011) 108:16277–82. doi:10.1073/pnas.1110286108
48. Tsai JA, Janson A, Bucht E, Kindmark H, Marcus C, Stark A, et al. Weak evidence of thyrotropin receptors in primary cultures of human osteoblast-like cells. *Calcif Tissue Int* (2004) 74:486–91. doi:10.1007/s00223-003-0108-3
49. Morimura T, Tsunekawa K, Kasahara T, Seki K, Ogiwara T, Mori M, et al. Expression of type 2 iodothyronine deiodinase in human osteoblast is stimulated by thyrotropin. *Endocrinology* (2005) 146:2077–84. doi:10.1210/en.2004-1432
50. Bagriacik EU, Yaman M, Haznedar R, Sucak G, Delibasi T. TSH-induced gene expression involves regulation of self-renewal and differentiation-related genes in human bone marrow-derived mesenchymal stem cells. *J Endocrinol* (2012) 212:169–78. doi:10.1530/JOE-11-0404
51. Ramajayam G, Vignesh RC, Karthikeyan S, Kumar KS, Karthikeyan GD, Veni S, et al. Regulation of insulin-like growth factors and their binding proteins by thyroid stimulating hormone in human osteoblast-like (SaOS2) cells. *Mol Cell Biochem* (2012) 368:77–88. doi:10.1007/s11010-012-1345-4
52. Boyce BF, Xing L. Osteoclasts, no longer osteoblast slaves. *Nat Med* (2006) 12:1356–8. doi:10.1038/nm1206-1356
53. Teitelbaum SL. Bone resorption by osteoclasts. *Science* (2000) 289:1504–8. doi:10.1126/science.289.5484.1504
54. Soysa NS, Alles N, Aoki K, Ohya K. Osteoclast formation and differentiation: an overview. *J Med Dent Sci* (2012) 59:65–74.
55. Abu-Amer Y. NF-kappaB signaling and bone resorption. *Osteoporos Int* (2013) 24:2377–86. doi:10.1007/s00198-013-2313-x
56. Ma R, Morshed S, Latif R, Zaidi M, Davies TF. The influence of thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies on osteoclastogenesis. *Thyroid* (2011) 21:897–906. doi:10.1089/thy.2010.0457
57. Zhang W, Zhang Y, Liu Y, Wang J, Gao L, Yu C, et al. Thyroid-stimulating hormone maintains bone mass and strength by suppressing osteoclast differentiation. *J Biomech* (2014) 47:1307–14. doi:10.1016/j.jbiomech.2014.02.015
58. Lam J, Takeshita S, Barker JE, Kanagawa O, Ross FP, Teitelbaum SL. TNF-alpha induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand. *J Clin Invest* (2000) 106:1481–8. doi:10.1172/JCI11176
59. Yamoah K, Brebene A, Baliram R, Inagaki K, Dolios G, Arabi A, et al. High-mobility group box proteins modulate tumor necrosis factor-alpha expression in osteoclastogenesis via a novel deoxyribonucleic acid sequence. *Mol Endocrinol* (2008) 22:1141–53. doi:10.1210/me.2007-0460
60. Dallas SL, Prideaux M, Bonewald LF. The osteocyte: an endocrine cell … and more. *Endocr Rev* (2013) 34:658–90. doi:10.1210/er.2012-1026
61. Bonewald LF, Johnson ML. Osteocytes, mechanosensing and Wnt signaling. *Bone* (2008) 42:606–15. doi:10.1016/j.bone.2007.12.224
62. Seeman E, Delmas PD. Bone quality – the material and structural basis of bone strength and fragility. *N Engl J Med* (2006) 354:2250–61. doi:10.1056/NEJMra053077
63. Han Y, Cowin SC, Schaffler MB, Weinbaum S. Mechanotransduction and strain amplification in osteocyte cell processes. *Proc Natl Acad Sci U S A* (2004) 101:16689–94. doi:10.1073/pnas.0407429101
64. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med* (2011) 17:1231–4. doi:10.1038/nm.2452
65. Cordier AC, Denef JF, Haumont SM. Thyroid gland in dwarf mice: a stereological study. *Cell Tissue Res* (1976) 171:449–57. doi:10.1007/BF00220237

66. Taylor BA, Rowe L. The congenital goiter mutation is linked to the thyroglobulin gene in the mouse. *Proc Natl Acad Sci U S A* (1987) 84:1986–90. doi:10.1073/pnas.84.7.1986
67. Beamer WG, Cresswell LA. Defective thyroid ontogenesis in fetal hypothyroid (hyt/hyt) mice. *Anat Rec* (1982) 202:387–93. doi:10.1002/ar.1092020311
68. Stein SA, Oates EL, Hall CR, Grumbles RM, Fernandez LM, Taylor NA, et al. Identification of a point mutation in the thyrotropin receptor of the hyt/hyt hypothyroid mouse. *Mol Endocrinol* (1994) 8:129–38. doi:10.1210/mend.8.2.8170469
69. Zhang M, Tong KP, Fremont V, Chen J, Narayan P, Puett D, et al. The extracellular domain suppresses constitutive activity of the transmembrane domain of the human TSH receptor: implications for hormone-receptor interaction and antagonist design. *Endocrinology* (2000) 141:3514–7. doi:10.1210/endo.141.9.7790
70. Sun L, Zhu LL, Lu P, Yuen T, Li J, Ma R, et al. Genetic confirmation for a central role for TNFalpha in the direct action of thyroid stimulating hormone on the skeleton. *Proc Natl Acad Sci U S A* (2013) 110:9891–6. doi:10.1073/pnas.1308336110
71. Marians RC, Ng L, Blair HC, Unger P, Graves PN, Davies TF. Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice. *Proc Natl Acad Sci U S A* (2002) 99:15776–81.
72. Sun L, Vukicevic S, Baliram R, Yang G, Sendak R, McPherson J, et al.. Intermittent recombinant TSH injections prevent ovariectomy-induced bone loss. *Proc Natl Acad Sci U S A* (2008) 105:4289–94. doi:10.1073/pnas.0712395105
73. Hase H, Ando T, Eldeiry L, Brebene A, Peng Y, Liu L, et al. TNFalpha mediates the skeletal effects of thyroid-stimulating hormone. *Proc Natl Acad Sci U S A* (2006) 103:12849–54. doi:10.1073/pnas.0600427103
74. Sun L, Davies TF, Blair HC, Abe E, Zaidi M. TSH and bone loss. *Ann N Y Acad Sci* (2006) 1068:309–18. doi:10.1196/annals.1346.033
75. Zaidi M, Sun L, Davies TF, Abe E. Low TSH triggers bone loss: fact or fiction? *Thyroid* (2006) 16:1075–6. doi:10.1089/thy.2006.16.1075
76. Svare A, Nilsen TI, Bjoro T, Forsmo S, Schei B, Langhammer A. Hyperthyroid levels of TSH correlate with low bone mineral density: the HUNT 2 study. *Eur J Endocrinol* (2009) 161:779–86. doi:10.1530/EJE-09-0139
77. Leader A, Ayzenfeld RH, Lishner M, Cohen E, Segev D, Hermoni D. Thyrotropin levels within the lower normal range are associated with an increased risk of hip fractures in euthyroid women, but not men, over the age of 65 years. *J Clin Endocrinol Metab* (2014) 99:2665–73. doi:10.1210/jc.2013-2474
78. Heemstra KA, van der Deure WM, Peeters RP, Hamdy NA, Stokkel MP, Corssmit EP, et al. Thyroid hormone independent associations between serum TSH levels and indicators of bone turnover in cured patients with differentiated thyroid carcinoma. *Eur J Endocrinol* (2008) 159:69–76. doi:10.1530/EJE-08-0038
79. Mazziotti G, Sorvillo F, Piscopo M, Cioffi M, Pilla P, Biondi B, et al. Recombinant human TSH modulates in vivo C-telopeptides of type-1 collagen and bone alkaline phosphatase, but not osteoprotegerin production in postmenopausal women monitored for differentiated thyroid carcinoma. *J Bone Miner Res* (2005) 20:480–6. doi:10.1359/JBMR.041126
80. Giusti M, Cecoli F, Ghiara C, Rubinacci A, Villa I, Cavallero D, et al. Recombinant human thyroid stimulating hormone does not acutely change serum osteoprotegerin and soluble receptor activator of nuclear factor-kappaBeta ligand in patients under evaluation for differentiated thyroid carcinoma. *Hormones (Athens)* (2007) 6:304–13. doi:10.14310/horm.2002.1111026
81. van der Deure WM, Uitterlinden AG, Hofman A, Rivadeneira F, Pols HA, Peeters RP, et al. Effects of serum TSH and FT4 levels and the TSHR-Asp727Glu polymorphism on bone: the Rotterdam Study. *Clin Endocrinol (Oxf)* (2008) 68:175–81. doi:10.1111/j.1365-2265.2007.03016.x
82. Britto JM, Fenton AJ, Holloway WR, Nicholson GC. Osteoblasts mediate thyroid hormone stimulation of osteoclastic bone resorption. *Endocrinology* (1994) 134:169–76. doi:10.1210/endo.134.1.8275930
83. Gothe S, Wang Z, Ng L, Kindblom JM, Barros AC, Ohlsson C, et al. Mice devoid of all known thyroid hormone receptors are viable but exhibit disorders of the pituitary-thyroid axis, growth, and bone maturation. *Genes Dev* (1999) 13:1329–41. doi:10.1101/gad.13.10.1329

Conflict of Interest Statement: TD is on the Board of Kronus Inc., Star, ID, USA. The remaining authors have nothing to disclose.

The reviewer IR and handling editor declared their shared affiliation.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 19 April 2017
doi: 10.3389/fendo.2017.00083

The Role of Thyrotropin Receptor Activation in Adipogenesis and Modulation of Fat Phenotype

Mohd Shazli Draman[1], Michael Stechman[2], David Scott-Coombes[2], Colin M. Dayan[1], Dafydd Aled Rees[3], Marian Ludgate[1] and Lei Zhang[1]**

[1]Thyroid Research Group, Cardiff University, Cardiff, UK, [2]Department of Endocrine Surgery, University Hospital of Wales, Cardiff, UK, [3]School of Medicine, Neuroscience and Mental Health Research Institute, Cardiff University, Cardiff, UK

Evidence from clinical and experimental data suggests that thyrotropin receptor (TSHR) signaling is involved in energy expenditure through its impact on white adipose tissue (WAT) and brown adipose tissue (BAT). TSHR expression increases during mesenchymal stem cell (MSC) differentiation into fat. We hypothesize that TSHR activation [TSHR*, elevated thyroid-stimulating hormone, thyroid-stimulating antibodies (TSAB), or activating mutation] influences MSC differentiation, which contributes to body composition changes seen in hypothyroidism or Graves' disease (GD). The role of TSHR activation on adipogenesis was first investigated using *ex vivo* samples. Neck fat (all euthyroid at surgery) was obtained from GD ($n = 11$, TSAB positive), toxic multinodular goiter (TMNG, TSAB negative) ($n = 6$), and control patients with benign euthyroid disease ($n = 11$, TSAB negative). The effect of TSHR activation was then analyzed using human primary abdominal subcutaneous preadipocytes ($n = 16$). Cells were cultured in complete medium (CM) or adipogenic medium [ADM, containing thiazolidinedione (TZD), PPARγ agonist, which is able to induce BAT formation] with or without TSHR activation (gain-of-function mutant) for 3 weeks. Adipogenesis was evaluated using oil red O (ORO), counting adipogenic foci, qPCR measurement of terminal differentiation marker (*LPL*). BAT [*PGC-1α*, uncoupling protein 1 (*UCP1*), and *ZIC1*], pre-BAT (*PRDM16*), BRITE– (*CITED1*), or WAT (*LEPTIN*) markers were analyzed by semiquantitative PCR or qPCR. In *ex vivo* analysis, there were no differences in the expression of *UCP1*, *PGC-1α*, and *ZIC1*. BRITE marker *CITED1* levels were highest in GD followed by TMNG and control (p for trend = 0.009). This was associated with higher WAT marker *LEPTIN* level in GD than the other two groups ($p < 0.001$). In primary cell culture, TSHR activation substantially enhanced adipogenesis with 1.4 ± 0.07 (ORO), 8.6 ± 1.8 (foci), and 5.5 ± 1.6 (*LPL*) fold increases compared with controls. Surprisingly, TSHR activation in CM also significantly increased pre-BAT marker *PRDM16*; furthermore, TZD-ADM induced adipogenesis showed substantially increased BAT markers, *PGC-1α* and *UCP1*. Our study revealed that TSHR activation plays an important role in the adipogenesis process and BRITE/pre-BAT formation, which leads to WAT or BAT phenotype. It may contribute to weight loss as heat during hyperthyroidism and later transforms into WAT posttreatment of GD when patients gain excess weight.

Keywords: adipogenesis, thyrotropin receptor, body composition, white adipose tissue, BRITE adipocytes, brown adipose tissue

Abbreviations: TSHR, thyrotropin receptor; MSC, mesenchymal stem cell; TSAB, thyroid-stimulating antibodies; CM, complete medium; ADM, adipogenic medium; WAT, white adipose tissue; BAT, brown adipose tissue.

OPEN ACCESS

Edited by:
Susanne Neumann, National Institutes of Health, USA

Reviewed by:
Ann Marie Zavacki, Brigham and Women's Hospital, USA
Pieter de Lange, Università degli Studi della Campania "Luigi Vanvitelli" Caserta, Italy

****Correspondence:***
Marian Ludgate
ludgate@cardiff.ac.uk;
Lei Zhang
zhangL14@cardiff.ac.uk

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *13 January 2017*
Accepted: *31 March 2017*
Published: *19 April 2017*

Citation:
Draman MS, Stechman M, Scott-Coombes D, Dayan CM, Rees DA, Ludgate M and Zhang L (2017) The Role of Thyrotropin Receptor Activation in Adipogenesis and Modulation of Fat Phenotype. Front. Endocrinol. 8:83. doi: 10.3389/fendo.2017.00083

INTRODUCTION

Excess thyrotropin receptor (TSHR) activation occurs in two common conditions, Graves' disease (GD) in which thyroid-stimulating antibodies (TSAB) mimic thyroid-stimulating hormone (TSH) causing hyperthyroidism and primary hypothyroidism when elevated circulating TSH compensates for low thyroid hormone (T4/T3) levels resulting from the failing gland (1). Both confer alterations in body composition, e.g., more than 90% of people with GD lose weight, mainly muscle mass and fat (2), while hypothyroidism increases fat and bone mineral density. The opposing differences of thyroid hormone levels have been traditionally suggested for these changes of body compositions, e.g., impact on white adipose tissue (WAT) or brown adipose tissue (BAT) metabolism (3, 4). BAT depots were thought to be absent from adult humans, but the availability of imaging using ^{18}F-fluorodeoxyglucose positron emission tomography and computed tomography reveals their location in supraclavicular and neck regions (5–7).

Despite restoration of serum TSH concentrations to normal, many GD patients complain of substantial weight gain post treatment (8, 9) with potential negative impact on their future cardiovascular risk. Further studies in this area may therefore have considerable impact on determining the optimal treatment for patients with GD. There is still considerable controversy regarding the best treatment for Graves' hyperthyroidism and radioiodine and/or thyroidectomy might be associated with more weight gain compared to those on antithyroid drugs and patients who undergo ablative therapy for thyroid cancer (10–12). This suggests that there are some factors associated with GD that influence post therapy weight gain. Furthermore, analysis has suggested that a diagnosis of GD (as opposed to other causes of thyroid over-activity) is an independent predictor of weight gain (10), raising the possibility that the persisting anti-TSH receptor antibodies in such patients might have long-term effects on peripheral adipose tissue composition (13). In humans, lipolysis was shown to be stimulated by TSH and TSAB, but this was confined to neonates suggesting an effect predominantly on BAT (14). Furthermore, the presence of functional extrathyroidal TSHR has been demonstrated in adipose tissue (15, 16) and bone (17), and fat-specific knockout of TSHR generated mice with larger adipocytes (18). More recent studies report a positive correlation between TSHR activation and obesity (19–21), and reports using animal models suggest a role for the TSHR in BAT and WAT function (22–24). For example, TSHR-deficient hyt/hyt mice became hypothermic in cold conditions, despite thyroxine administration, but transfection of TSHR into BAT of these mice improved core temperature (22). The above evidence led us to hypothesize that TSHR activation *per se* may contribute to changes in body composition separately from the effects of thyroid hormone levels, exerting a direct impact on adipose tissues metabolism (25).

Brown adipose tissue dissipates energy as heat (thermogenesis) in a process mediated by uncoupling protein 1 (UCP1), which uncouples oxidative phosphorylation from ATP production (26). WAT and BAT are derived from distinct lineages of mesenchymal stem cells (MSCs), Myf5+ for BAT (also muscle progenitors) but Myf5− for WAT (27). The two adipose types also differ morphologically with WAT having a single large fat vacuole and BAT having many smaller fat droplets and higher numbers of mitochondria (28). In addition to WAT and newly documented adult BAT (5), human beige (or BRITE for BRown in whITE) adipocytes have been recently identified, and like WAT are derived from Myf5− MSC (29). Although there are clearly defined BAT, BRITE, and WAT depots in mice, human fat depots tend to be heterogeneous with BRITE/pre-BAT adipocytes present in both WAT and inducible BAT depots with the potential to be transformed to either WAT or BAT (30, 31). Transcription factor *PRDM16* plays an essential role in the transformation of BRITE/pre-BAT to BAT (32). Adipocytes are generated by lineage-specific differentiation of MSC found in fat (33); the expression of TSHR is increased in human fat depots undergoing adipogenesis (34). We hypothesize that TSHR activation could thus modulate fat formation. Our aim was to investigate the effect of TSHR activation on human adipose tissue from the neck, which is recognized as a BAT inducible region (5), by phenotyping *ex vivo* samples using markers for WAT, BRITE, and BAT. Furthermore, we analyzed preadipocytes obtained from subcutaneous adipose tissue, to address the role of TSHR activation in adipogenesis and modulation of fat phenotype.

MATERIALS AND METHODS

All reagents were obtained from Sigma-Aldrich and tissue culture components from Cambrex unless otherwise stated.

Adipose Tissue Collection

Subcutaneous adipose tissue ($n = 16$) was collected from patients undergoing elective open abdominal surgery for non-metabolic conditions. For *ex vivo* analysis, subcutaneous neck fat samples were obtained from GD ($n = 11$), toxic multinodular goiter (TMNG) ($n = 6$) and euthyroid control patients with benign thyroid nodules ($n = 11$) undergoing thyroid surgery. Five patients have undetectable TSH measurements (GD = 4 and TMNG = 1) with normal free T4 (one at upper limit normal level) or T3 levels. The suppressed TSH is expected in treated hyperthyroid patients as this will take months to recover despite being euthyroid. It should be stressed that all patients were clinically euthyroid during surgical procedure and patients' information has been summarized in **Table 1**. All GD patients have positive TSHR antibodies measured by thyroid-binding inhibiting immunoglobulin assays and TSAB luciferase reporter assay (35).

Generation of TSHR*-Expressing Cells

Preadipocyte/fibroblasts were obtained by collagenase digest, as previously described (36). Cells were used at low passage number (<5); hence, not all samples were analyzed in all experiments. Activating mutant TSHR (L629F) was introduced into the preadipocyte populations using retroviral vectors, previously produced in our laboratory (37). Geneticin selection resulted in mixed populations stably expressing the various TSHR, which exhibit increased basal levels of cAMP compared with the equivalent non-modified cell population, all as previously described (16).

TABLE 1 | Patients demographic.

Patient ID	Sex	Age	Hist	FT3	FT4	TSH	TRAB	TPO	TSAB	EUT (months)
GD										
GD1	M	47	GD		13.0	2.06	32		2.6	8
GD2	F	71	GD	4.7	11.4	6.83	4.7	<2	2.9	7
GD3	F	23	GD		7.1	7.84	<1	1,059	3.0	7
GD4	M	48	GD	4.3	14.9	0.04	11.7	<2	2.4	4
GD5	F	63	GD		22.4	<0.02	6.7		2.5	3
GD6	F	39	GD	5.6	17.5	<0.02			3.1	6
GD7	F	52	GD	5.6	9.3	0.29	15.8	>1,000	3.0	21
GD8	F	38	GD		13.2	0.43			2.9	12
GD9	M	31	GD		12.3	2.34				3
GD10	F	57	TMNG	4.5	12.9	<0.02	19.3	648	2.7	10
GD11	F	27	TMNG	4.6	14.1	<0.02			3.3	6
Toxic MNG										
MNG1	F	43	TMNG	5.3	17.8	<0.02			1.6	2
MNG2	M	76	TMNG		13.0	0.1	<1		1.1	9
MNG3	F	61	TMNG		14.3	0.92		50	1.2	20
MNG4	F	70	TA		13.0	1.22	<1	12.5	1.2	16
MNG5	M	61	TMNG		13.5	0.21			1.2	21
MNG6	M	89	TMNG		14.1	0.25			1.1	9
Control										
CO1	F	21	CN		13.5	1.56			1.4	
CO2	F	78	HN		13.0	3.26			1.1	
CO3	F	46	BC		12.6	1.46			1.2	
CO4	F	71	EMNG		16.7	1.79			1.3	
CO5	M	50	EMNG		12.2	0.61			1.1	
CO6	F	27	EMNG		14.0	2.58		<2	1.2	
CO7	F	78	EMNG		13.7	0.11			1.8	
CO8	F	27	EMNG		14.6	1.09			1.2	
CO9	F	61	EMNG		14.0	0.57		<2	1.3	
CO10	M	45	EMNG		16.5	0.83		<2	1.4	
CO11	F	71	EMNG		13.0	0.65		300	1.2	

F, female; M, male; Hist, histology; EUT, euthyroid duration; GD, Graves' disease; TMNG, toxic multinodular goiter; EMNG, euthyroid multinodular goiter; TA, toxic adenoma; CN, colloid nodules; HN, hyperplastic nodules; BC, benign cyst.

Normal reference: FT3 (2.6–5.7 pmol/l), FT4 (9.2–22 pmol/l), thyroid-stimulating hormone (TSH) (0.30–4.40 mU/l), thyroid receptor antibodies (TRAB; <1 negative, 1–1.4, borderline >1.4 U/l positive), thyroid peroxidase antibodies (TPO) (<32 kU/l is negative), and thyroid-stimulating antibodies (TSAB; stimulation index 97.5th SD of normal <1.4 is negative).

Preadipocyte/Fibroblast Culture *In Vitro* Adipogenesis

Preadipocytes were cultured in DMEM/F12 10% FCS (complete medium, CM). Adipogenesis was induced in confluent cells by replacing with differentiation medium [adipogenic medium (ADM)] containing 10% FCS, biotin (33μM), panthothenate (17μM), T3 (1nM), dexa-methasone (100nM), thiazolidinedione (TZD) (1μM), and insulin (500nM) for 22 days, adipogenesis was assessed by microscopic examination to detect the characteristic morphological changes (cell rounding, accumulation of lipid droplets), acquisition of lipid filled droplets [oil red O (ORO) staining], and transcript measurement of adipogenic markers (*PPARγ*, *LPL*) by qPCR as described previously (16). In addition, foci of differentiation (groups of cells with lipid droplets) were counted in 10 different fields for each experimental condition (36).

PCR Analysis of Markers for WAT, BRITE, or BAT

Transcript copy numbers for various genes, including markers for WAT [*LEPTIN* (38)], BRITE [*CITED1* (39)], pre-BAT (*PRDM16*), and BAT [*PGC-1α*, *UCP1*, and *ZIC1* (30, 31)] together with *TSHR* were measured.

Total RNA from cells or *ex vivo* fat tissues was extracted and reverse transcribed using standard protocols (16) for standard or qPCR analysis; primers (cross exon boundaries to avoid amplification of genomic DNA) were designed using primer 3 software (Table S1 in Supplementary Material). qPCR was conducted using SYBR Green incorporation measured on a Stratagene MX 3000. Comparison with plasmid standard curves for each gene permitted calculation of absolute values for each sample (transcripts per microgram input RNA). In addition, for qPCR, transcripts of a housekeeping gene, *APRT*, were measured so that values could be expressed relative to this (transcripts/1,000 *APRT*). *APRT* was also used in the comparative Ct method to assess transcript levels of *PRDM16*. It should be noted that none of the treatments used resulted in a variation in the *APRT* Ct value of more than one cycle. In qPCR experiments, all measurements were made in triplicate; the standard curve was also run in at least duplicate.

If multiple products (e.g., primer dimer) were detected by qPCR (dissociation curve), a classic PCR with densitometry technique was used. Standard PCR was performed to detect *CITED1* and *LEPTIN* using Phusion High-Fidelity PCR master mix (Thermo Scientific) as per the manufacturer's instructions. The PCR products were resolved on 2% agarose gels for 35 min,

and densitometry values were obtained and corrected to housekeeping gene (*GAPDH*).

Statistical Analysis

Parametric data were analyzed using Student's *t*-test and one-way ANOVA for multiple group comparisons where appropriate. Similarly, Mann–Whitney *U* test and Kruskal–Wallis *H* test was used for non-parametric data. All analysis was done using two-tailed tests. Parametric data were presented as mean ± SD and median ± interquartile range for non-parametric data. In all cases, $p < 0.05$ was considered significant.

RESULTS

TSHR Activation Favor BRITE and WAT Formation in *Ex Vivo* Analysis

To examine the role of TSHR activation on adipose tissues, we analyzed markers of WAT, BRITE, and BAT using *ex vivo* samples of subcutaneous neck fat. Analyzed samples were obtained when patients were euthyroid, but the persisting TSAB result in GD fat samples experiencing ongoing TSHR activation while TMNG and control samples do not.

Expression levels of *TSHR* did not differ in control, TMNG, or GD groups (**Figure 1A**); we then analyzed the potential effect of TSAB/TSHR* on fat phenotype. Higher transcript levels of *LEPTIN* (WAT marker) were detected in GD samples compared to TMNG and control ($p < 0.001$) (**Figure 1C**), which indicates that adipogenesis in the WAT compartment is ongoing in GD. BAT markers *PGC-1α*, *UCP1*, and *ZIC1* were detected, even though there was no difference in expression levels between the three groups (**Figure 1B**). However, a well-defined BRITE marker, *CITED1*, showed highest transcript levels in GD samples, followed by TMNG and control (p value, test for trend = 0.0009) (**Figure 1D**).

These data suggest that TSHR activation is associated with WAT and BRITE fat generation; subsequently, we tried to understand the role of TSHR activation on adipogenesis using primary cell cultures.

TSHR Activation Enhances Subcutaneous Adipogenesis Induced *In Vitro*

We first investigated the effects of TSHR signaling on adipogenesis using subcutaneous preadipocytes stably expressing or not activating mutant TSHR (L629F, TSHR*). TSHR activation did not induce spontaneous adipogenesis in subcutaneous

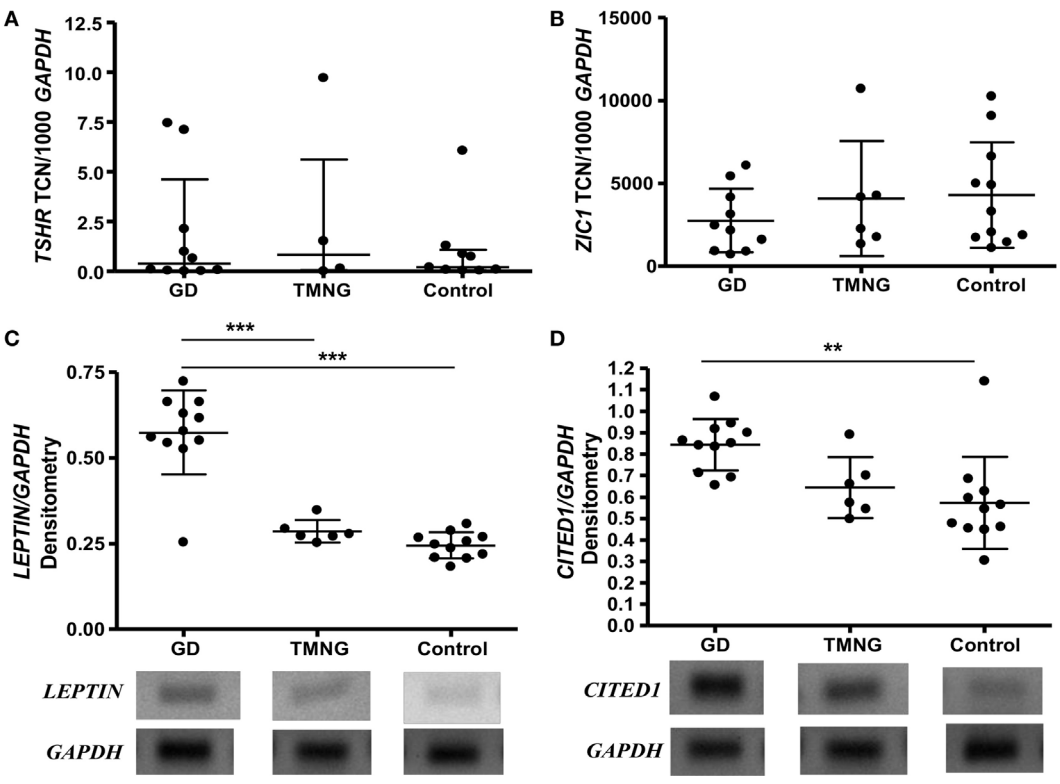


FIGURE 1 | *Ex vivo* analysis of deep neck fat. Samples were snap frozen, and total RNA was isolated. Gene transcripts were measured by qPCR, **(A)** thyrotropin receptor (*TSHR*); **(B)** *ZIC1* (brown adipose tissue marker), shown as transcript copy number (TCN) per 1,000 copies of housekeeper gene *APRT* (adenosine phosphoribosyl transferase); standard PCR was used to analyze **(C)** *LEPTIN* (white adipose tissue marker), **(D)** *CITED1* (BRITE marker), densitometry were measured and corrected to housekeeping gene (*GAPDH*). Representative photos were shown (gels with all samples had been included in Figure S1 in Supplementary Material). Post-ANOVA test for linear trend of *CITED1* was performed ($p = 0.009$). Results expressed as mean ± SD of all samples studied (each performed in duplicate) (**$p \leq 0.01$; ***$p < 0.001$).

precursors even when the cells were examined for morphological signs up to 3 weeks after reaching confluence. In contrast, after 22 days incubation in an adipogenic cocktail, we observed substantial enhancement of this lineage-specific differentiation by TSHR activation, whether assessed morphologically, by semiquantitative ORO staining or qPCR measurement of transcripts for *LPL* (marker of terminal differentiation) as shown in **Figure 2**.

We concluded that TSHR activation enhances *in vitro*-induced adipogenesis. The adipogenic cocktail (ADM) used in this study contains PPARγ agonist TZD, which is known to stimulate BAT formation (40). Consequently, we conducted experiments to understand the impact of TSHR activation on BAT formation both in basal and induced adipogenesis conditions.

TSHR Activation Enhanced BAT Formation of Subcutaneous Precursors

We selected several markers including pre-BAT *PRDM16*, BAT *PGC-1α* (transcriptional regulator of BAT formation), and *UCP1* (terminal BAT marker) (29).

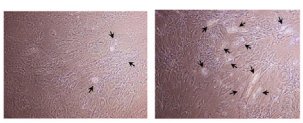
These were measured in subcutaneous preadipocytes on day 0 in CM and following *in vitro*-induced adipogenesis in TZD-ADM for 22 days.

On day 0 (basal condition), the cells experiencing TSHR activation displayed substantially higher transcript levels of the pre-BAT marker, *PRDM16,* when compared with the control population as shown in **Figure 3A**. However, TSHR activation had no significant effect on expression levels of *PGC-1α* and *UCP1* (**Figures 3B,C**).

By contrast, at day 22 following TZD-ADM induced adipogenesis, TSHR activation significantly increased transcript levels of *PGC-1α* and *UCP1* when compared with control cells in ADM conditions but lacking TSHR activation (**Figures 3B,C**).

DISCUSSION

Our study suggests that TSHR activation enhances adipogenesis and could contribute to the modulation of fat phenotype.



	EMP	TSHR*	Fold changes
Counting foci	8 ± 3.2	39.6 ± 10.1 ***	8.6 ± 1.8
LPL/1000 APRT	201.6 ± 120.6	611.1 ± 299.6 *	5.5 ± 1.6
ORO	0.22 ± 0.02	0.3 ± 0.04 **	1.4 ± 0.07

FIGURE 2 | Adipogenesis in subcutaneous preadipocytes was assessed by foci counting ($n = 11$, representative photos were shown with arrows indicating differentiating adipocytes), LPL transcripts measured by qPCR ($n = 9$) and oil red O (ORO, $n = 4$). Result presented as fold increase in TSHR* populations relative to empty vector controls. The table reports raw data for qPCR results expressed as transcript copy number per 1,000 copies of housekeeper gene *APRT* (adenosine phosphoribosyl transferase), together with foci numbers and ORO optical density values (mean ± SEM). Histograms = mean ± SEM of all samples studied (each performed in duplicate) (*$p < 0.05$; **$p \leq 0.01$; ***$p < 0.005$).

The *ex vivo* data demonstrate that in samples of fat from TSAB positive GD patients, transcript levels of markers for WAT (*LEPTIN*) and BRITE (*CITED1*) were significantly higher than in corresponding samples from people with TMNG or controls (all TSAB negative). Since all patients were euthyroid at the time of surgery, it is reasonable to conclude that the TSAB have a role in the observed effect and the presence of TSHR transcripts in all samples confirms that this would be plausible.

Having access to samples of neck adipose tissue is fortuitous since Cypess and colleagues reported a gradient from the surface to the midline of WAT *via* BRITE to BAT in this region using biomarkers, e.g., *LEPTIN* (WAT), gradient *UCP1* expression (BRITE to BAT) or *ZIC* (41, 42). Studies in mice suggest a bidirectional interconversion of BRITE and white adipocytes (43), with BRITE adipocytes being induced by cold temperature into brown fat and conversely returning into white fat at higher temperatures. Similarly, Lee et al. found that BRITE fat can be transformed into brown or white by adrenergic stimulation and high-calorie diet, respectively (44). High thyroid hormone levels are known to induce brown fat activity in BAT and BRITE fat (3, 4, 45). Of note, the samples from the GD and TMNG patients would have previously encountered a period of thyroid hormone excess, during the hyperthyroid phase of their condition but only in GD would there have been simultaneous TSHR activation.

We hypothesize that in GD, TSHR activation increases adipogenesis and, combined with excess thyroid hormone, favors formation of BAT. This could also explain the heat intolerance of GD patients, which is usually attributed to excess thyroid hormone increasing metabolic rate. The concept is further supported by studies in the hyt/hyt mouse, which lacks a functional TSHR and deals poorly with low temperature, a characteristic which can be overcome by transfecting WT TSHR into the animals (22). Proof could be provided by comparing expression of WAT, BRITE, and BAT markers in adipose tissue from TSAB-positive GD patients when hyperthyroid and then when euthyroidism is restored; however, patient safety during surgery precludes this.

Our *in vitro* studies confirmed that TSHR activation increases adipogenesis in subcutaneous fat, in contrast to orbital fat, in which we have previously reported that TSHR activation inhibits induced adipogenesis (16). These effects are the opposite of the situation in hyperthyroid GD in which the majority of fat stores are depleted, with the exception of orbital fat which expands in some patients. Our studies have demonstrated that orbital adipogenesis is under differing regulatory mechanisms compared with non-orbital fat precursors (46, 47).

We also investigated whether TSHR activation had any effect on fat phenotype *in vitro*, we recognize that there may be some overlap in markers for WAT, BAT, pre-BAT, and BRITE but have selected the best characterized for each (48). We found that subcutaneous precursors, experiencing TSHR activation, had significantly higher transcript levels of the pre-BAT marker, *PRDM16* in basal conditions. There were no other indicators of spontaneous adipogenesis in these cells. In ADM, TSHR activation significantly increased differentiation and also significantly increased expression levels of BAT markers *PGC*-1 and *UCP1*.

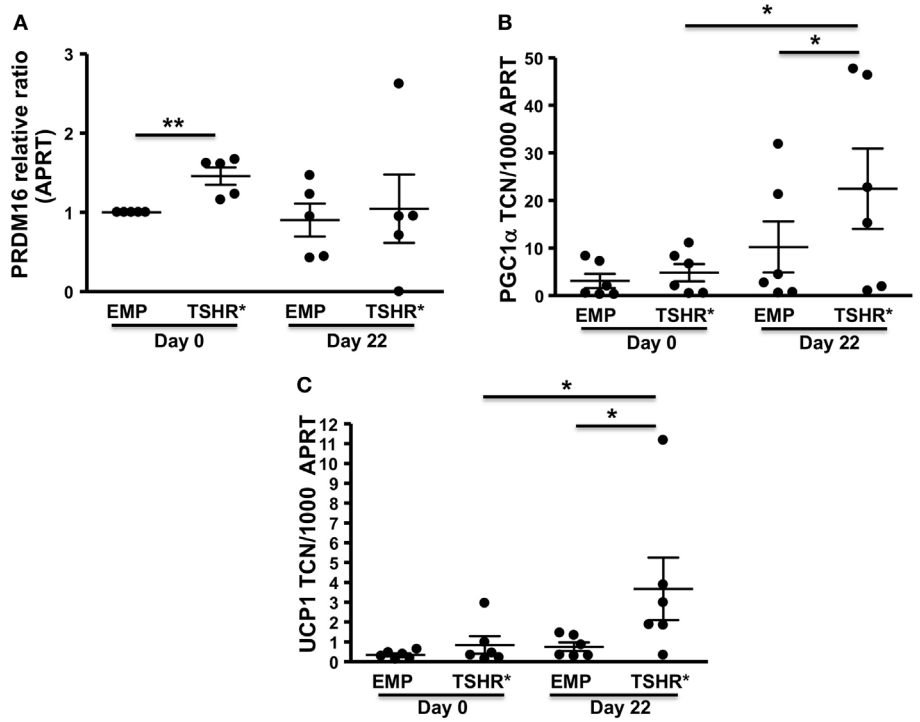


FIGURE 3 | Preadipocytes/fibroblasts from subcutaneous fat ($n = 6$) expressing activating thyrotropin receptor (TSHR) mutant L629F (TSHR*) and equivalent empty vector controls were cultured until confluent (day 0) before changing to differentiation medium for 22 days. Total RNA was isolated before or after adipogenesis. PRDM16 (relative expression ratio) **(A)**, *PGC-1*α **(B)**, and uncoupling protein 1 (*UCP1*) **(C)** transcripts were measured by qPCR. Results are expressed as comparative qPCR (relative ratio to APRT) of PRDM16 or absolute qPCR (*PGC-1*α and *UCP1*) of transcript copy number (TCN) per 1,000 copies of housekeeper gene *APRT* (adenosine phosphoribosyl transferase). Histograms = mean ± SEM of all samples studied (each performed in duplicate). Two-tailed *t*-test or Mann–Whitney test used for statistic analysis (*$p < 0.03$; **$p = 0.01$).

Our findings are similar to those of Cypess et al. who induced adipogenesis in the presence of Db-cAMP and obtained significant enhancement of *PGC-1α* and *UCP1* compared with control cells in ADM alone (41). In our *in vitro* model, cells experiencing TSHR activation (mutant TSHR) display a twofold to fourfold increase in basal cAMP when compared to empty vector control cells (16).

The *in vitro* findings support our hypothesis that TSHR activation enhances adipogenesis and favors BAT formation in the hyperthyroid state. Our *ex vivo* findings imply that enhanced adipogenesis persists but the fat phenotype is WAT with some features of BRITE rather than BAT. Could this contribute to the weight gain experienced in GD following treatment? If increased adipogenesis produces BAT, energy can be dissipated as heat (as in GD) and weight will be shed. Once the BAT phenotype is lost (posttreatment of GD), then the increased adipogenesis would lead to WAT accumulation and weight will be gained.

Current therapy is based on inhibiting thyroid hormone production medically, surgically, or using radioiodine ablation. Future strategies aimed at neutralizing TSAB, for example, using TSHR antagonist could be more effective in solving weight problem.

ETHICS STATEMENT

The South East Wales Research Ethics committee approved this study; all fat samples were collected with informed consent, and written consent was obtained.

AUTHOR CONTRIBUTIONS

LZ and MD performed majority of the experiments; LZ, ML, and MD wrote the manuscript. DR and CD obtained ethical approval and reviewed the manuscript. MS, DS-C, and MD obtained fat samples from patients. LZ and ML coordinated the project, data analysis, and writing of the manuscript.

FUNDING

The authors are grateful to the National Eye Research Centre that partly supported this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fendo.2017.00083/full#supplementary-material.

REFERENCES

1. Paschke R, Ludgate M. The thyrotropin receptor in thyroid diseases. *N Engl J Med* (1997) 337:1675–81. doi:10.1056/NEJM199712043372307
2. Seppel T, Kosel A, Schlaghecke R. Bioelectrical impedance assessment of body composition in thyroid disease. *Eur J Endocrinol* (1997) 136:493–8. doi:10.1530/eje.0.1360493
3. Lahesmaa M, Orava J, Schalin-Jantti C, Soinio M, Hannukainen JC, Noponen T, et al. Hyperthyroidism increases brown fat metabolism in humans. *J Clin Endocrinol Metab* (2014) 99:E28–35. doi:10.1210/jc.2013-2312
4. Obregon MJ. Adipose tissues and thyroid hormones. *Front Physiol* (2014) 5:479. doi:10.3389/fphys.2014.00479
5. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med* (2009) 360:1509–17. doi:10.1056/NEJMoa0810780
6. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* (2009) 360:1500–8. doi:10.1056/NEJMoa0808718
7. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. *N Engl J Med* (2009) 360:1518–25. doi:10.1056/NEJMoa0808949
8. Tigas S, Idiculla J, Beckett G, Toft A. Is excessive weight gain after ablative treatment of hyperthyroidism due to inadequate thyroid hormone therapy? *Thyroid* (2000) 10:1107–11. doi:10.1089/thy.2000.10.1107
9. Jansson S, Berg G, Lindstedt G, Michanek A, Nystrom E. Overweight – a common problem among women treated for hyperthyroidism. *Postgrad Med J* (1993) 69:107–11. doi:10.1136/pgmj.69.808.107
10. Dale J, Daykin J, Holder R, Sheppard MC, Franklyn JA. Weight gain following treatment of hyperthyroidism. *Clin Endocrinol* (2001) 55:233–9. doi:10.1046/j.1365-2265.2001.01329.x
11. Pears J, Jung RT, Gunn A. Long-term weight changes in treated hyperthyroid and hypothyroid patients. *Scott Med J* (1990) 35:180–2. doi:10.1177/003693309003500609
12. Weinreb JT, Yang Y, Braunstein GD. Do patients gain weight after thyroidectomy for thyroid cancer? *Thyroid* (2011) 21:1339–42. doi:10.1089/thy.2010.0393
13. Laurberg P, Wallin G, Tallstedt L, Abraham-Nordling M, Lundell G, Torring O. TSH-receptor autoimmunity in Graves' disease after therapy with anti-thyroid drugs, surgery, or radioiodine: a 5-year prospective randomized study. *Eur J Endocrinol* (2008) 158:69–75. doi:10.1530/EJE-07-0450
14. Marcus C, Ehren H, Bolme P, Arner P. Regulation of lipolysis during the neonatal period. Importance of thyrotropin. *J Clin Invest* (1988) 82:1793–7. doi:10.1172/JCI113793
15. Crisp MS, Lane C, Halliwell M, Wynford-Thomas D, Ludgate M. Thyrotropin receptor transcripts in human adipose tissue. *J Clin Endocrinol Metab* (1997) 82:2003–5.
16. Zhang L, Baker G, Janus D, Paddon CA, Fuhrer D, Ludgate M. Biological effects of thyrotropin receptor activation on human orbital preadipocytes. *Invest Ophthalmol Vis Sci* (2006) 47:5197–203. doi:10.1167/iovs.06-0596
17. Abe E, Marians RC, Yu W, Wu XB, Ando T, Li Y, et al. TSH is a negative regulator of skeletal remodeling. *Cell* (2003) 115:151–62. doi:10.1016/S0092-8674(03)00771-2
18. Elgadi A, Zemack H, Marcus C, Norgren S. Tissue-specific knockout of TSHR in white adipose tissue increases adipocyte size and decreases TSH-induced lipolysis. *Biochem Biophys Res Commun* (2010) 393:526–30. doi:10.1016/j.bbrc.2010.02.042
19. Lu S, Guan Q, Liu Y, Wang H, Xu W, Li X, et al. Role of extrathyroidal TSHR expression in adipocyte differentiation and its association with obesity. *Lipids Health Dis* (2012) 11:17. doi:10.1186/1476-511X-11-17
20. Ma S, Jing F, Xu C, Zhou L, Song Y, Yu C, et al. Thyrotropin and obesity: increased adipose triglyceride content through glycerol-3-phosphate acyltransferase 3. *Sci Rep* (2015) 5:7633. doi:10.1038/srep07633
21. Muscogiuri G, Sorice GP, Mezza T, Prioletta A, Lassandro AP, Pirronti T, et al. High-normal TSH values in obesity: is it insulin resistance or adipose tissue's guilt? *Obesity (Silver Spring)* (2013) 21:101–6. doi:10.1002/oby.20240
22. Endo T, Kobayashi T. Thyroid-stimulating hormone receptor in brown adipose tissue is involved in the regulation of thermogenesis. *Am J Physiol Endocrinol Metab* (2008) 295:E514–8. doi:10.1152/ajpendo.90433.2008
23. Endo T, Kobayashi T. Expression of functional TSH receptor in white adipose tissues of TSH receptor mutant mice induces lipolysis in vivo. *Am J Physiol Endocrinol Metab* (2012) 302:E1569–75. doi:10.1152/ajpendo.00572.2011
24. Martinez-deMena R, Anedda A, Cadenas S, Obregon MJ. TSH effects on thermogenesis in rat brown adipocytes. *Mol Cell Endocrinol* (2015) 404:151–8. doi:10.1016/j.mce.2015.01.028
25. de Lloyd A, Bursell J, Gregory JW, Rees DA, Ludgate M. TSH receptor activation and body composition. *J Endocrinol* (2010) 204:13–20. doi:10.1677/JOE-09-0262
26. Chondronikola M, Volpi E, Borsheim E, Porter C, Annamalai P, Enerback S, et al. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes* (2014) 63:4089–99. doi:10.2337/db14-0746
27. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, et al. PRDM16 controls a brown fat/skeletal muscle switch. *Nature* (2008) 454:961–7. doi:10.1038/nature07182
28. Stephens M, Ludgate M, Rees DA. Brown fat and obesity: the next big thing? *Clin Endocrinol* (2011) 74:661–70. doi:10.1111/j.1365-2265.2011.04018.x
29. Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev* (2013) 27:234–50. doi:10.1101/gad.211649.112
30. Wu J, Bostrom P, Sparks LM, Ye L, Choi JH, Giang AH, et al. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell* (2012) 150:366–76. doi:10.1016/j.cell.2012.05.016
31. Jespersen NZ, Larsen TJ, Peijs L, Daugaard S, Homoe P, Loft A, et al. A classical brown adipose tissue mRNA signature partly overlaps with brite in the supraclavicular region of adult humans. *Cell Metab* (2013) 17:798–805. doi:10.1016/j.cmet.2013.04.011
32. Cohen P, Levy JD, Zhang Y, Frontini A, Kolodin DP, Svensson KJ, et al. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. *Cell* (2014) 156:304–16. doi:10.1016/j.cell.2013.12.021
33. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* (2002) 13:4279–95. doi:10.1091/mbc.E02-02-0105
34. Starkey KJ, Janezic A, Jones G, Jordan N, Baker G, Ludgate M. Adipose thyrotrophin receptor expression is elevated in Graves' and thyroid eye diseases ex vivo and indicates adipogenesis in progress in vivo. *J Mol Endocrinol* (2003) 30:369–80. doi:10.1677/jme.0.0300369
35. Evans C, Morgenthaler NG, Lee S, Llewellyn DH, Clifton-Bligh R, John R, et al. Development of a luminescent bioassay for thyroid stimulating antibodies. *J Clin Endocrinol Metab* (1999) 84:374–7. doi:10.1210/jcem.84.1.5532
36. Rice SP, Zhang L, Grennan-Jones F, Agarwal N, Lewis MD, Rees DA, et al. Dehydroepiandrosterone (DHEA) treatment in vitro inhibits adipogenesis in human omental but not subcutaneous adipose tissue. *Mol Cell Endocrinol* (2010) 320:51–7. doi:10.1016/j.mce.2010.02.017
37. Fuhrer D, Lewis MD, Alkhafaji F, Starkey K, Paschke R, Wynford-Thomas D, et al. Biological activity of activating thyroid-stimulating hormone receptor mutants depends on the cellular context. *Endocrinology* (2003) 144:4018–30. doi:10.1210/en.2003-0438
38. Ussar S, Lee KY, Dankel SN, Boucher J, Haering MF, Kleinridders A, et al. ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. *Sci Transl Med* (2014) 6:247ra103. doi:10.1126/scitranslmed.3008490
39. Sharp LZ, Shinoda K, Ohno H, Scheel DW, Tomoda E, Ruiz L, et al. Human BAT possesses molecular signatures that resemble beige/brite cells. *PLoS One* (2012) 7:e49452. doi:10.1371/journal.pone.0049452
40. Ohno H, Shinoda K, Spiegelman BM, Kajimura S. PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. *Cell Metab* (2012) 15:395–404. doi:10.1016/j.cmet.2012.01.019
41. Cypess AM, White AP, Vernochet C, Schulz TJ, Xue R, Sass CA, et al. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nat Med* (2013) 19:635–9. doi:10.1038/nm.3112
42. Nedergaard J, Cannon B. How brown is brown fat? It depends where you look. *Nat Med* (2013) 19:540–1. doi:10.1038/nm.3187

43. Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. *Nat Cell Biol* (2013) 15:659–67. doi:10.1038/ncb2740
44. Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. *Cell Metab* (2012) 15:480–91. doi:10.1016/j.cmet.2012.03.009
45. Lee JY, Takahashi N, Yasubuchi M, Kim YI, Hashizaki H, Kim MJ, et al. Triiodothyronine induces UCP-1 expression and mitochondrial biogenesis in human adipocytes. *Am J Physiol Cell Physiol* (2012) 302:C463–72. doi:10.1152/ajpcell.00010.2011
46. Zhang L, Grennan-Jones F, Lane C, Rees DA, Dayan CM, Ludgate M. Adipose tissue depot-specific differences in the regulation of hyaluronan production of relevance to Graves' orbitopathy. *J Clin Endocrinol Metab* (2012) 97:653–62. doi:10.1210/jc.2011-1299
47. Zhang L, Ji QH, Ruge F, Lane C, Morris D, Tee AR, et al. Reversal of pathological features of Graves' orbitopathy by activation of forkhead transcription factors, FOXOs. *J Clin Endocrinol Metab* (2016) 101:114–22. doi:10.1210/jc.2015-2932
48. Peirce V, Carobbio S, Vidal-Puig A. The different shades of fat. *Nature* (2014) 510:76–83. doi:10.1038/nature13477

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 25 July 2016
doi: 10.3389/fendo.2016.00103

Bioassays for TSH Receptor Autoantibodies, from FRTL-5 Cells to TSH Receptor–LH/CG Receptor Chimeras: The Contribution of Leonard D. Kohn

Cesidio Giuliani[1], Motoyasu Saji[2], Ines Bucci[1] and Giorgio Napolitano[1]*

[1] Unit of Endocrinology, Department of Medicine and Sciences of Aging, Ce.S.I.-Me.T., University of Chieti–Pescara, Chieti, Italy, [2] Department of Internal Medicine, Division of Endocrinology, Diabetes and Metabolism, The Ohio State University, Columbus, OH, USA

OPEN ACCESS

Edited by:
Yuji Nagayama, Nagasaki University, Japan

Reviewed by:
Akira Hishinuma, Dokkyo Medical University, Japan
Takao Ando, Nagasaki University Hospital, Japan

***Correspondence:**
Cesidio Giuliani
cgiulian@unich.it

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *20 May 2016*
Accepted: *12 July 2016*
Published: *25 July 2016*

Citation:
Giuliani C, Saji M, Bucci I and Napolitano G (2016) Bioassays for TSH Receptor Autoantibodies, from FRTL-5 Cells to TSH Receptor–LH/CG Receptor Chimeras: The Contribution of Leonard D. Kohn. Front. Endocrinol. 7:103. doi: 10.3389/fendo.2016.00103

Since the discovery 60 years ago of the "long-acting thyroid stimulator" by Adams and Purves, great progress has been made in the detection of thyroid-stimulating hormone (TSH) receptor (TSHR) autoantibodies (TRAbs) in Graves' disease. Today, commercial assays are available that can detect TRAbs with high accuracy and provide diagnostic and prognostic evaluation of patients with Graves' disease. The present review focuses on the development of TRAbs bioassays, and particularly on the role that Leonard D. Kohn had in this. Indeed, 30 years ago, the Kohn group developed a bioassay based on the use of FRTL-5 cells that was characterized by high reproducibility, feasibility, and diagnostic accuracy. Using this FRTL-5 bioassay, Kohn and his colleagues were the first to develop monoclonal antibodies (moAbs) against the TSHR. Furthermore, they demonstrated the multifaceted functional nature of TRAbs in patients with Graves' disease, with the identification of stimulating and blocking TRAbs, and even antibodies that activated pathways other than cAMP. After the cloning of the TSHR, the Kohn laboratory constructed human TSHR–rat luteinizing hormone/chorionic gonadotropin receptor chimeras. This paved the way to a new bioassay based on the use of non-thyroid cells transfected with the Mc4 chimera. The new Mc4 bioassay is characterized by high diagnostic and prognostic accuracy, greater than for other assays. The availability of a commercial kit based on the Mc4 chimera is spreading the use of this assay worldwide, indicating its benefits for these patients with Graves' disease. This review also describes the main contributions made by other researchers in TSHR molecular biology and TRAbs assay, especially with the development of highly potent moAbs. A comparison of the diagnostic accuracies of the main TRAbs assays, as both immunoassays and bioassays, is also provided.

Keywords: TSH receptor bioassay, FRTL-5 cells, Graves' disease, TSHR autoantibodies, chimera

INTRODUCTION

Thyroid-stimulating hormone (TSH) receptor (TSHR) autoantibodies (TRAbs) are the pathogenic hallmark of Graves' disease. They are detected in nearly all untreated patients with Graves' disease and are responsible for the pathological features of this disease (i.e., stimulation of thyroid growth and function, onset of orbitopathy, and/or dermopathy) (1). Several varieties of TRAbs have been described: stimulating (TSAbs), blocking (TBAbs), and neutral (N-TRAbs). Their relative concentrations define the clinical picture and the progression of Graves' disease. Indeed, quantitation of TRAbs is of clinical use not only to confirm the diagnosis of Graves' disease but also to predict the evolution of the disease and its complications, such as orbitopathy (2, 3). Furthermore, TBAbs are involved in the pathogenesis of hypothyroidism in the atrophic form of Hashimoto's thyroiditis (4).

Since the discovery by Adams and Purves in 1956, of a thyroid-stimulating factor in the serum of some thyrotoxic patients (5), remarkable progress has been made in the knowledge of the biological properties of TRAbs. Furthermore, very sensitive assays are now commercially available to detect TRAbs (**Figure 1**). The purpose of this article is to review the development of these TRAbs bioassays, with a focus on the contributions made here by the late Dr. Leonard D. Kohn.

HISTORICAL BACKGROUND

In 1956, Adams and Purves noted that sera from thyrotoxic patients induced abnormal prolonged responses in their TSH bioassay that used guinea pigs (5, 6). They initially named the unknown substance that was responsible for this effect as the "abnormal thyroid stimulator," and then later as the "long-acting thyroid stimulator" (LATS) (6, 7). Soon after its discovery, it became apparent that this LATS was distinct from endogenous TSH and that it was not produced by the pituitary (7). In 1964, LATS was identified as a protein with the biological characteristics of an antibody (8), and further studies unequivocally demonstrated its identification with immunoglobulin G (IgG) (9, 10).

The early *in vivo* bioassays to detect LATS were performed using guinea pigs or mice, but these were of little use in clinical practice as they were troublesome and had very low sensitivity. Indeed, 30–40% of patients with Graves' disease were negative with these assays (11). A significant breakthrough was then made in 1975, with the development of a radioligand receptor assay, which evaluated the inhibition by the sera from patients with Graves' disease of the binding of radiolabeled TSH to human thyroid membranes *in vitro* (12). However, this assay was still burdened by low accuracy. Further improvements to the method were provided by the use of the partially purified TSHR instead of thyroid membranes and biologically active radiolabeled TSH. This thus led to the development of a reproducible and accurate radioligand assay some years later (13, 14). This assay has been defined as a liquid phase first-generation immunoassay, and it was widely used for the next 20 years. It had a specificity of 99.2% (range, 97.5–100%) and a sensitivity of 79.8% (range, 52–94%) (15).

In parallel with the development of the radioligand receptor assay, there was also an improvement in the bioassay methods, with the replacement of the *in vivo* assay with *in vitro* techniques, such as the use of thyroid slices or thyroid primary cell cultures (16). A further fundamental advance was obtained with the development of FRTL-5 cells, a non-transformed cell line of rat thyroid epithelial cells in continuous culture (17). Indeed, the Kohn laboratory at the National Institutes of Health in Bethesda used these FRTL-5 cells to set up an accurate assay for the measurement of TSAbs, which provided greater convenience and reproducibility compared to other bioassays (18–20). From that time, FRTL-5 cells became the preferred tool for TRAbs bioassays for more than 10 years, and as discussed below, they were fundamental to the determination and quantification of the functional properties of TRAbs.

THE FRTL-5 BIOASSAY

FRTL-5 cells are a cell line that can be grown in continuous culture and that retains all of the properties of normal thyroid

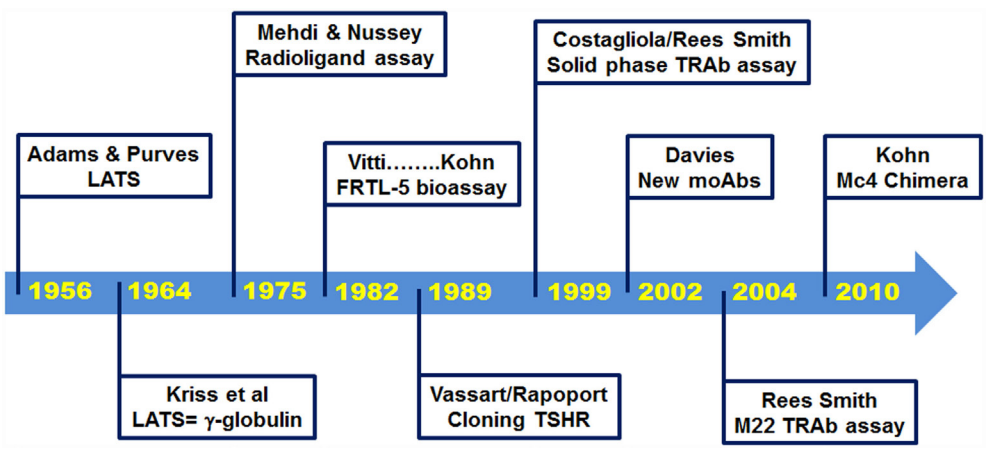


FIGURE 1 | Milestones in history of TSH receptor antibody assays.

cells. Soon after their development, the Kohn group described the optimal conditions to measure TSAbs using FRTL-5 cells (18, 19). The assay was based on the ability of purified IgG preparations to induce cAMP production. Removal of TSH from the culture medium resulted in an enhanced response to acute stimulation by TSH and TSAbs. This assay showed a specificity of 97.6% and a sensitivity of 90.4%, thus providing a sensitivity that exceeded that of the liquid phase first-generation immunoassay (19, 21). The assay method was patented (22), and this paved the way to the commercial availability of the bioassay, and to its spread. Of note, all of the royalties associated with this patent were dispensed in the forms of grants to international researchers in the field of thyroidology. A further improvement in the feasibility of this test was provided with the direct use of the patient sera, rather than the purified IgG (23).

This FRTL-5 bioassay was not only important for diagnostic purposes but also a fundamental tool in the characterization of the functional properties of TRAbs and the understanding of their pathogenic role in Graves' disease. The Kohn laboratory was particularly active in pursuing this. Indeed, it was Kohn and his colleagues who first developed monoclonal antibodies (moAbs) against TSHR, and they used the FRTL-5 cells to evaluate their functional properties (24–26). The generation of moAbs from lymphocytes of patients with Graves' disease was also of significance, as this demonstrated the multifaceted functional nature of TRAbs, with some stimulating and others blocking the receptor activity (25, 26). These data were of great importance for the confirmation of TBAbs in Graves' disease, as had been postulated previously (27, 28).

The use of the FRTL-5 cells also provided the possibility to further study the functional heterogeneity of TRAbs, as they allowed the separate assessment of the effects of an individual IgG on two distinct cellular activities: those of the production of cAMP and of cell growth. Indeed, Kohn and colleagues performed both cAMP assays and thymidine-incorporation assays in cells incubated with sera from patients with Graves' disease. Through this, they demonstrated that these patients with Graves' disease fell into one of three groups (**Figure 2**): those where the IgGs had strong cAMP-stimulating activity together with strong growth-promoting activity (group 1); those where the IgGs had strong growth-promoting activity, but little or no cAMP-stimulating activity (group 2); and those where the IgGs had strong cAMP-stimulating activity, but low growth-promoting activity (group 3) (29). This study demonstrated the separate and distinct effects of TRAbs on cAMP production and cell growth, which suggested that other transduction mechanisms as well as cAMP might be involved in their interactions with TSHR. This assumption was later confirmed by several studies, most of which were performed in the Kohn laboratory, which showed that the growth and function of thyroid cells were dependent on the ability of TSH to activate not only cAMP signaling but also other signaling pathways, such as those of phospholipase C and phospholipase A_2/arachidonic acid (30, 31). A further confirmation came from studies, which showed that a subpopulation of IgGs from patients with Graves' disease activated the phospholipase A_2 pathway without affecting the cAMP signal (32, 33). These studies were fundamental to the correlation of the clinical heterogeneity of Graves' disease with its pathogenesis.

Today, the heterogeneity of TRAbs is well recognized, and in addition to the classical TSAbs and TBAbs, which act as TSH agonists and antagonists, respectively, other forms of TRAbs have been described, in terms of the neutral antibodies (N)-TRAbs

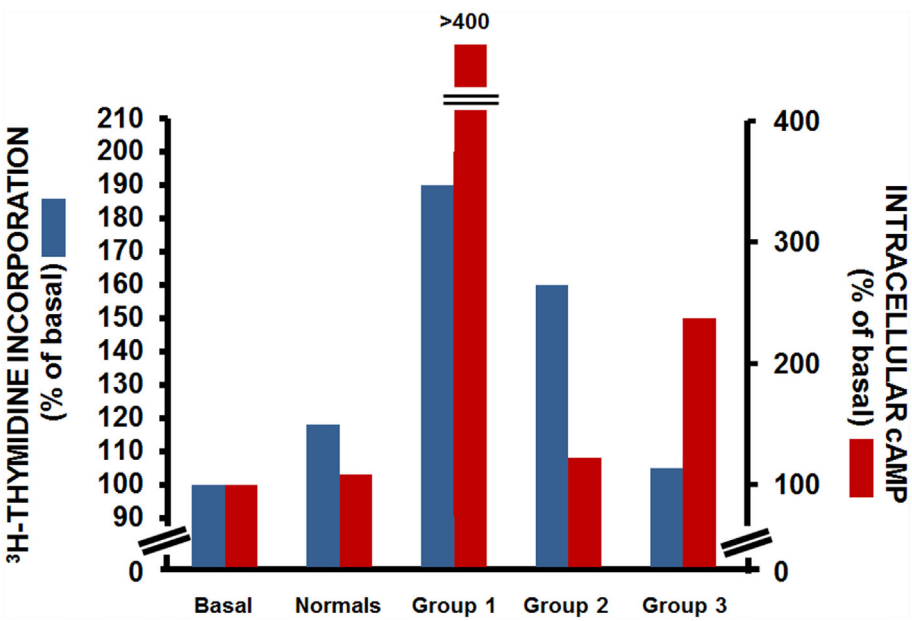


FIGURE 2 | Effects of IgG from patients with Graves' disease on [³H]-thyimidine incorporation (blue) and intracellular cAMP production (red) in FRTL-5 cells. Data from three representative patients with Graves' disease for each group. Basal, cells with no treatment; normals, cells incubated with a pooled sample of IgG from 13 non-Graves' individuals [Data are from Ref. (29)].

(**Table 1**). The N-TRAbs are so called because their binding to TSHR does not influence the binding of TSH and the cAMP levels, although they can activate other signaling cascades (1, 34, 35). Moreover, some of the antibodies that have been regarded as TBAbs have shown some growth-promoting activity independent of cAMP signaling (36).

THE CLONING OF THE TSH RECEPTOR

A major breakthrough in thyroid research arrived with the cloning of TSHR in 1989 (37–39). The cloning allowed the use of the recombinant human (rh-)TSHR, both for the radioligand receptor assay and the bioassay (40, 41). This led to an increase in the sensitivity of the radioligand receptor assay to 96% (41), which was higher than that of the FRTL-5 bioassay. The cloning also improved the feasibility of the use of the bioassay, as it was possible to transfect rh-TSHR into non-thyroid cell lines that were characterized by simpler culture conditions than the FRTL-5 cells (42, 43). The new transfected rh-TSHR bioassay was also characterized by better sensitivity than the FRTL-5 bioassay. Indeed, a comparative study performed using purified IgGs from 58 patients with Graves' disease showed that a bioassay based on Chinese hamster ovary (CHO) cells transfected with rh-TSHR had a higher sensitivity than the FRTL-5 bioassay (93 vs. 75.8%, respectively) (42). These data were confirmed by an independent study that showed a similar sensitivity for these two bioassays (92.2 and 74.5%, respectively) (43).

TABLE 1 | Summary of the functional characteristics of TRAbs.

Antibody	Effect on TSH binding	Effect on cAMP levels	Interference with cAMP-independent signaling
Stimulating	Inhibition	Increase	Yes
Blocking	Inhibition	Inhibition	Yes
Neutral	No effect	No effect	Yes

Moreover, the cloning of TSHR led to a series of studies that were mainly based on site-directed mutagenesis, deletion mutants, and the construction of receptor chimeras, which provided the pioneering achievements in the structure–function relationships of TSHR (31, 44, 45). The Kohn group was particularly involved in these studies, and in particular, in the construction of human TSHR–rat luteinizing hormone/chorionic gonadotropin receptor (TSHR–LH/CGR) chimeras (46), as these paved the way to the new TRAbs bioassays. A series of TSHR–LH/CGR chimeras were then constructed by replacing the homologous segments of the extracellular domain of the human TSHR with the corresponding segments of the rat LH/CGR, and these were used to identify receptor binding sites for TSH and TRAbs. Two chimeras were of particular interest and are known as the Mc1 + 2 and the Mc4 chimeras (**Figure 3**). The Mc1 + 2 chimera has a large portion of the N-terminal extracellular region of TSHR substituted (amino-acid residues 8–165), and it retains TSH binding and TSH stimulation of cAMP levels. However, the Mc1 + 2 chimera does not have the TSAbs activity, i.e., TSAbs cannot stimulate cAMP production or inhibit TSH binding to the chimera. However, its TBAbs binding affinity is maintained (**Table 2**). The Mc4 chimera has amino-acid residues 261–370 substituted, and it retains the ability for TSH and TSAbs binding and to still promote increased cAMP levels, whereas it no longer shows TBAbs binding (**Table 2**). These data suggested that the TRAbs that show different functional activities, i.e., TSAbs and TBAbs, have epitopes that are located in distinct regions of the extracellular domain of TSHR. More precisely, TSAbs are largely directed against the N-terminus region of TSHR, which includes amino-acid residues 8–165, whereas TBAbs

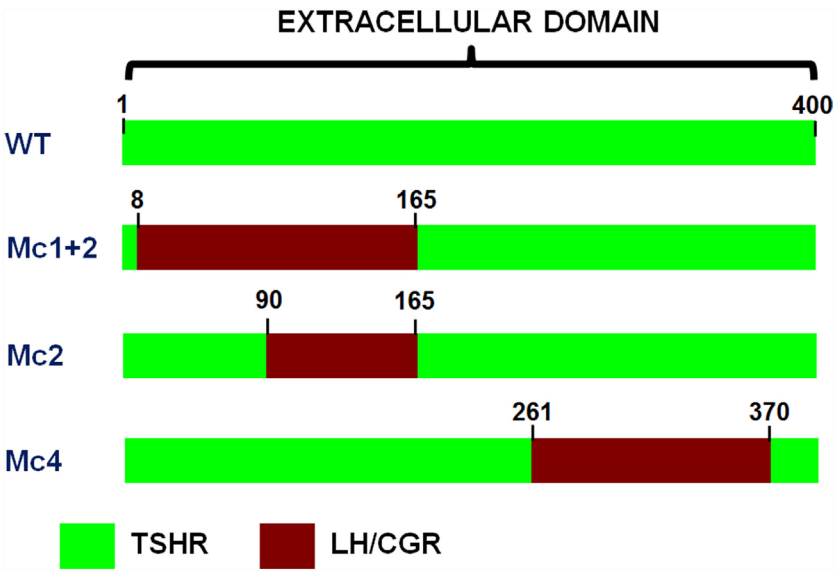


FIGURE 3 | Schematic representations of the extracellular regions of the three chimeric TSHR–LH/CGR mutants. The Mc1 + 2, Mc2, and Mc4 chimera receptors. Green, human TSHR sequence (WT); red, homologous regions of the rat LH/CGR that were used to substitute for the deleted TSHR sequences. The numbers assigned to the amino acids are determined by counting from the methionine start site.

TABLE 2 | Summary of functional properties of TSHR–LH/CGR chimeras.

Receptor/chimera	TSH binding	TSAb binding	TBAb binding
TSHR wild-type	Yes	Yes	Yes
Mc1 + 2	Yes	No	Yes
Mc2	Yes	No	Yes
Mc4	Yes	Yes	No

mainly bind the C-terminal region, which includes amino-acid residues 261–370 (46, 47). Similar data were obtained simultaneously by the Rapoport group, who also demonstrated some degree of overlap between the epitopes for TSAbs and TBAbs (45, 48). Subsequent studies then showed significant overlap among these epitopes, while also describing more of their complex characteristics (49–51). However, the functional data described above induced Kohn to establish a new bioassay based on the use of these TSHR–LH/CGR chimeras to evaluate the clinical relevance of autoantibody heterogeneity in patients with Graves' disease.

THE TSHR–LH/CGR CHIMERA BIOASSAY

The clinical use of the TRAbs bioassay based on the chimeric receptor was evaluated using CHO cells stably transfected with the rh-TSHR, the Mc1 + 2 TSHR–LH/CGR chimera described above, or the Mc2 chimera, in which residues 90–165 of the TSHR ectodomain were substituted (**Figure 3**). A preliminary study that was performed using purified IgG from 66 patients with Graves' disease showed that although the TSAbs activities in the majority of patients was not detectable in the cells transfected with the Mc1 + 2 or Mc2 chimeras, in approximately 30% of the patients there were TSAbs that could activate these chimeric receptors (52). Therefore, these patients with Graves' disease could be divided into two groups: a homogeneous group, with TSAbs that recognized only the N-terminal region of the TSHR ectodomain and did not activate the chimeric receptors, and a heterogeneous group, with TSAbs that interacted with the C-terminal region of the TSHR ectodomain and activated the chimeric receptors.

A very interesting observation came from the clinical correlation of these data. The heterogeneous group was more responsive to antithyroid therapies, which meant that the patients in this group were more likely to become euthyroid during treatment, and to do so more quickly. Moreover, a following study demonstrated that antithyroid drug therapies induced epitope heterogeneity, namely, during antithyroid treatment, about 50% of the patients with Graves' disease who were initially negative in the chimera assay became positive (53). These data were confirmed in a larger study that was characterized by a longer follow-up, which indicated that heterogeneity of TSAbs is a good and independent marker for prediction of the clinical outcome of patients with Graves' disease after antithyroid drug therapies (54).

IMPROVEMENT OF THE TRAbs ASSAY: NEW GENERATIONS OF IMMUNOASSAYS AND BIOASSAYS

In the late 1990s, a second generation of immunoassays was developed using moAbs against the C-terminus region of rh-TSHR or porcine TSHR. Plastic surfaces coated with these moAbs were used to immobilize TSHR, which was still able to bind TSH and TRAbs (55, 56).

This second-generation immunoassay, which is known as a "solid phase" assay, became the gold standard assay for TRAbs due to the high diagnostic accuracy and the use of a non-radioactive readout (15, 55–58). Indeed, a seminal study by Costagliola et al. (55) performed on 328 patients with Graves' disease showed the high sensitivity and specificity of this assay (98.8 and 99.6%, respectively), with no differences between the radioactive or chemiluminescence readouts. The use of rh-TSHR or porcine TSHR did not affect the diagnostic accuracy of the assay (57). These data were confirmed by subsequent studies, as reported in a recent meta-analysis (15). Given this high diagnostic accuracy and the availability of a commercial kit, the "solid phase" assay became the most used assay for the detection of TRAbs.

At the same time, several researchers were involved in the generation of moAbs against TSHR characterized by TSAb activity (59–61). Due to the availability of rh-TSHR for both animal immunization and antibody screening, highly potent moAbs were obtained that were characterized by their higher affinities (reaching the order of nM concentrations), compared with the previous moAbs, where the concentrations used were in the order of μM or mM (24–26). The Davies group was particularly involved in these studies, and they used the moAbs as molecular probes to investigate further the structure–function relationships of TSHR and its interactions with TRAbs (1, 35, 62–65). A number of new insights came from these studies: (1) TSAbs and most TBAbs recognize conformational epitopes in the α subunit of TSHR (i.e., involving the first 316 amino acids), with these epitopes either distinct or overlapping; (2) some TBAbs bind epitopes in the N-terminus of the β subunit of TSHR; (3) N-TRAbs bind linear epitopes that are mainly in the cleavage region; (4) TSHR is present on the cell surface in both its cleaved and uncleaved forms, and it can exit as multimers; (5) As opposed to TSH, TSAbs do not accelerate the cleavage of TSHR, and this might explain the prolonged overstimulation of the thyroid gland in Graves' patients; and (6) N-TRAbs can activate alternative signal pathways to the classical cAMP pathway. These data have been fundamental in the understanding the structure–function relationships of TSHR and its role in the pathogenesis of Graves' disease. Furthermore, in 2003, this research on these moAbs led to the isolation and characterization of the human monoclonal TSAb M22 from lymphocytes of a patient with Graves' disease (66).

A third-generation immunoassay was then developed based on the use of this M22 autoantibody (67). Indeed, given its high affinity binding to TSHR, the labeled M22 autoantibody was then used instead of labeled bovine TSH in inhibition assays, with significant improvements to the intra-assay coefficient of variation (15, 58). This new immunoassay became the preferred TRAbs assay due to its high diagnostic accuracy and feasibility. Indeed, the pooled sensitivity from all of the data reported in the literature is 97.4% (range, 95–99.6%), and the pooled specificity is 99.2% (range, 95–100%). Furthermore, the M22 assay is based on an ELISA method, and this is available also in a fully automated version (15, 68).

This availability of both stimulating (e.g., M22) and blocking human moAbs has also been useful for determination of the crystal structure of TSHR and its interactions with the TRAbs (49, 69). These studies confirmed the extensive overlap among the epitopes for TSAbs and TBAbs.

Concurrent with the development of the third-generation immunoassay, Kohn conceived the use of the Mc4 chimera (**Figure 2**) for a new bioassay. As indicated above, the Mc4 chimera retains the binding of TSH and TSAbs and the consequent activity but loses TBAbs binding (**Table 2**). Aside from arguments about different TSAbs and TBAbs epitopes, which as discussed above is a complex issue, several studies have provided the basis for the use of the Mc4 chimera, as reviewed by Lytton and Kahaly in 2010 (70). Further support for the use of the Mc4 chimera was provided by the finding that the shed A-subunit of the TSHR (spanning from approximately amino-acid residue 22–216), rather than the TSHR holoreceptor, is important for immunogenicity and for maturation affinity of TRAbs (71–74).

This new bioassay is based on a chemiluminescent method, as described by Watson and colleagues (75), which uses cell lines that are stably transformed with a reporter plasmid that contains the firefly luciferase gene under the transcriptional control of multiple cAMP-responsive elements. These transformed cell lines were transfected with the Mc4 chimera (**Figure 4**) and were evaluated using sera from patients with Graves' disease and other thyroid diseases, and normal subjects (76). The primary goal here was to create a bioassay that measured only TSAbs, without interference of the other TRAbs, and to have a clear cutoff between patients with Graves' disease and the controls. For this purpose, the Mc4 assay was compared with a bioassay using wild-type TSHR and with a second-generation immunoassay. This study showed that the Mc4 assay has higher sensitivity and specificity (i.e., 100 and 98.5%, respectively) than the compared assays (**Table 3**). Furthermore, the Mc4 assay showed even higher sensitivity than the third-generation M22 immunoassay, although with a little less specificity (**Table 3**). The high diagnostic accuracy of the Mc4 assay can be attributed to the lack of interference by TBAbs and N-TRAbs. Indeed, contrary to what is observed with the conventional bioassay using wild-type TSHR, sera from patients with idiopathic myxedema, who have high TBAbs activity, did not inhibit the TSAb activity of the sera in the Mc4 bioassay (76).

An important conclusion that came from this study of Giuliani et al. (76) was that, given its high diagnostic accuracy, the Mc4 assay can be used as a first-level test in the diagnosis of Graves' disease. However, given the almost similar diagnostic accuracy and the better feasibility, the M22 immunoassay remains the preferred TRAbs assay worldwide to date. On the other hand, the specific detection of TSAbs without interference of other antibodies directed against TSHR makes the Mc4 assay potentially useful in the follow-up of patients with Graves' disease. Indeed, one of the clinical problems of Graves' disease is the high possibility of relapse within the first 2 years after withdrawal of medical therapy (at approximately 50%). Therefore, the results of a prospective study are of particular interest, where the Mc4 assay was shown to be a sensitive index of remission and relapse in patients with Graves' disease (77). This study was performed in patients with Graves' disease treated with antithyroid drugs

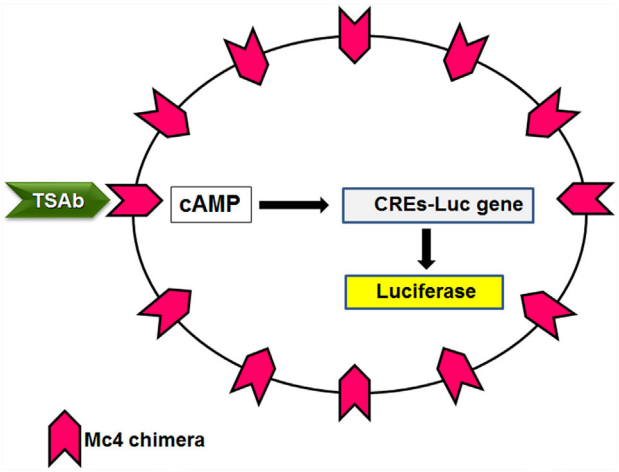


FIGURE 4 | Principle of the Mc4 bioassay. Cell lines lacking TSHR are double transfected with a luciferase reporter gene under the transcriptional control of multiple cAMP-responsive elements (CREs-Luc) and the Mc4 chimeric receptor. TSAbs levels in patient sera are determined by measuring the increased production of luciferase.

TABLE 3 | Comparison among sensitivity and specificity of the main TRAbs assays.

TRAbs assays	Sensitivity (%)	Specificity (%)
Mc4 bioassay	100[a]	98.5[a]
CHO–wtTSHR bioassay	97.3[a]	93.1[a]
"Solid phase" immunoassay	86.5[a]	97[a]
M22 ELISA assay	97.4[b]	99.2[b]

[a]*Data are from Ref. (76).*
[b]*Data are from Ref. (15).*

(mainly methimazole) over a 5-year period, and it showed that the levels of TSAbs correlate with the clinical outcome of the disease. Furthermore, here, the Mc4 assay had high accuracy as a predictor of Graves' disease prognosis, which was even better than the M22 third-generation immunoassay. A reasonable explanation for this is that the measure of only TSAbs instead of the whole spectrum of the TRAbs improves the prediction of the patient prognosis. Hence, the Mc4 assay might become a useful tool in identifying at an early stage those patients who will have no benefit from the medical therapy and to whom alternative therapeutic options can be offered. Indeed, failure to reduce TSAbs levels during medical therapy is a negative predictor of remission.

Retrospective studies have replicated the use of the Mc4 assay as a better indicator of the prognosis of patients with Graves' disease than these other assays (77, 78). Furthermore, several studies have shown that the Mc4 bioassay strongly correlates with the indices of clinical activity and severity of Graves' orbitopathy and has higher diagnostic accuracy than these other TRAbs assays (2, 70, 79–83). Indeed, a seminal study by Lytton et al. (79) showed that, compared with the second-generation immunoassays, the Mc4 assay had greater sensitivity (97 vs. 77%, respectively) and specificity (89 vs. 43%, respectively) for the detection of TRAbs in patients with Graves' orbitopathy. Furthermore, this study

demonstrated a strong correlation of TSAbs with the clinical activities of orbitopathy. Of interest, there was the observation that all patients whose sera were positive in the Mc4 assay and negative in the TRAbs immunoassay had severe orbitopathy, whereas those patients who tested negative with the Mc4 assay and positive with the TRAbs assay did not have active orbitopathy. These data confirm the superiority of the Mc4 assay in the detection of the subtypes of TRAbs that are directly involved in the pathogenesis of Graves' disease, without the interference of the other subtypes, such as blocking or neutral TRAbs, that have little or no pathogenic role in the clinical manifestations of Graves' disease. The studies that followed further confirmed these results and showed the usefulness of the Mc4 assay as a predictor of the clinical course of Graves' orbitopathy (2, 80, 82).

A recent multicenter study (81) showed that the Mc4 assay is more sensitive than the third-generation immunoassay in diagnosing Graves' disease in an untreated pediatric population. Moreover, as previously demonstrated in adult patients, the correlation of the Mc4 assay with the clinical activity and severity of Graves' orbitopathy was higher than seen for the third-generation immunoassay in these pediatric patients.

Widespread use of this bioassay will be facilitated by the availability of the Mc4 assay as a commercial kit, which has a standardized protocol and good feasibility and reproducibility (84, 85). Indeed, using the commercial kit, the bioassay can be performed in less than 24 h (70), and the concentrations determined can be converted in IU/L, with the possibility to standardize the TSAbs levels across laboratories, which provide more accurate comparisons of TSAbs levels (84). Of note, recently, the Mc4 chimera has been used to develop a new *in vitro* assay by applying Bridge technology (86). In brief, this Bridge Assay uses two TSH chimeric receptors: the Mc4 chimera, which is used as a capture receptor that is anchored on a solid phase, to bind one arm of the autoantibody; and a chimeric receptor formed by the N-terminus (aminoacids 21–261) of TSHR fused with secretory alkaline phosphatase as a chemiluminescence monitor, which can bind the other arm of the autoantibody. Preliminary data show good sensitivity and specificity for this Bridge Assay (99.8 and 99.5%, respectively) (86).

CONCLUSION

Thyroid-stimulating hormone receptor autoantibodies bioassays have several advantages in comparison to inhibition immunoassays. Bioassays can detect the functional heterogeneity of TRAbs in patients with Graves' disease; i.e., the simultaneous presence in the same patient of TSAbs, TBAbs, and/or N-TRAbs. This has clinical implications, because the switching from TSAbs to TBAbs is responsible for the evolution toward hypothyroidism in a small percentage of patients with Graves' disease. Moreover, a selective decrease in TSAbs is a positive prognostic feature for patient remission. Evaluation of TSAbs is also important in pregnant woman with Graves' disease, to estimate the risk of fetal/neonatal thyrotoxicosis due to TRAbs transfer. Finally, the monitoring of the switch from TSAbs to TBAbs, and *vice versa*, is very useful in patients with alternate episodes of hyperthyroidism and hypothyroidism.

In the past 60 years, TRAbs bioassays have evolved from cumbersome and time-consuming procedures to genetically engineered cell-based assays that are characterized by good feasibility and rapid operating times, and that are also available as commercial kits. The role that Kohn had in this process through all of these years was fundamental. Indeed, initially, the use of the FRTL-5 bioassay, and then the later generation of the Mc4 bioassay, led to striking progress in both the knowledge of the functional features of TRAbs and the clinical application of TRAbs bioassays. Kohn perceived the advantages that the use of the Mc4 chimera would bring in diagnostic accuracy and prognostic evaluation for patients with Graves' disease. He devoted himself to the improvement of the feasibility of the Mc4 bioassay to promote its use in clinical practice (85). The availability of the Mc4 bioassay as a commercial kit is now spreading the use of this assay worldwide. We believe that the improved feasibility of the Mc4 assay, together with its high diagnostic accuracy and prognostic use, will now make the Mc4 assay the preferred assay for clinical evaluation of patients with Graves' disease.

AUTHOR CONTRIBUTIONS

CG: substantial contributions to the conception and design of the work; drafting the work; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. MS: substantial contributions to the conception of the work; revising the work critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. IB: substantial contributions to the design of the work; revising the work critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. GN: substantial contributions to the conception of the work; revising it critically for intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Chris Berrie for linguistic revision of the manuscript.

REFERENCES

1. Michalek K, Morshed SA, Latif R, Davies TF. TSH receptor autoantibodies. *Autoimmun Rev* (2009) 9:113–6. doi:10.1016/j.autrev.2009.03.012

2. Jang SY, Shin DY, Lee EJ, Lee SY, Yoon JS. Relevance of TSH-receptor antibody levels in predicting disease course in Graves' orbitopathy: comparison of the third-generation TBII assay and Mc4-TSI bioassay. *Eye (Lond)* (2013) 27:964–71. doi:10.1038/eye.2013.120

3. Bartalena L, Burch HB, Burman KD, Kahaly GJ. A 2013 European survey of clinical practice patterns in the management of Graves' disease. *Clin Endocrinol* (2016) 84:115–20. doi:10.1111/cen.12688
4. Giuliani C, Monaco F. Adult primary hypothyroidism. In: Monaco F, editor. *Thyroid Diseases*. Boca Raton, FL: CRC Press (2012). p. 171–94.
5. Adams DD, Purves HD. Abnormal responses in the assay of thyrotropin. *Proc Univ Otago Med Sch* (1956) 34:11–5.
6. Adams DD. The presence of an abnormal thyroid-stimulating hormone in the serum of some thyrotoxic patients. *J Clin Endocrinol Metab* (1958) 18:699–712. doi:10.1210/jcem-18-7-699
7. Adams DD. Bioassay of long-acting thyroid stimulator (LATS); the dose-response relationship. *J Clin Endocrinol Metab* (1961) 21:799–805. doi:10.1210/jcem-21-7-799
8. Kriss JP, Pleshakov V, Chien JR. Isolation and identification of the long-acting thyroid stimulator and its relation to hyperthyroidism and circumscribed pretibial myxedema. *J Clin Endocrinol Metab* (1964) 24:1005–28. doi:10.1210/jcem-24-10-1005
9. Kriss JP. Inactivation of long-acting thyroid stimulator (LATS) by anti-kappa and anti-lamba antisera. *J Clin Endocrinol Metab* (1968) 28:1440–4. doi:10.1210/jcem-28-10-1440
10. Volpé R. The immunologic basis of Graves's disease. *N Engl J Med* (1972) 287:463–4. doi:10.1056/NEJM197208312870910
11. McKenzie JM. Review: pathogenesis of Graves' disease: role of the long-acting thyroid stimulator. *J Clin Endocrinol Metab* (1965) 25:424–31. doi:10.1210/jcem-25-3-424
12. Mehdi SQ, Nussey SS. A radio-ligand receptor assay for the long-acting thyroid stimulator. Inhibition by the long-acting thyroid stimulator of the binding of radioiodinated thyroid stimulating hormone to human thyroid membranes. *Biochem J* (1975) 145:105–11. doi:10.1042/bj1450105
13. Shewring G, Rees Smith B. An improved radioreceptor assay for TSH receptor antibodies. *Clin Endocrinol* (1982) 17:409–17. doi:10.1111/j.1365-2265.1982.tb01607.x
14. Southgate K, Creagh FM, Teece M, Kingswood C, Rees Smith B. A receptor assay for the measurement of TSH receptor antibodies in unextracted serum. *Clin Endocrinol* (1984) 20:539–48. doi:10.1111/j.1365-2265.1984.tb00102.x
15. Tozzoli R, Bagnasco M, Giavarina D, Bizzarro N. TSH receptor autoantibody immunoassay in patients with Graves' disease: improvement of diagnostic accuracy over different generations of methods, systematic review and meta-analysis. *Autoimmun Rev* (2012) 12:107–13. doi:10.1016/j.autrev.2012.07.003
16. Toccafondi RS, Aterini S, Medici MA, Rotella CM, Tanini A, Zonefrati R. Thyroid-stimulating antibody (TSAb) detected in sera of Graves' patients using human thyroid cell cultures. *Clin Exp Immunol* (1980) 40:532–9.
17. Ambesi-Impiombato FS, Parks LAM, Coon HG. Culture of hormone-dependent functional epithelial cells from rat thyroids. *Proc Natl Acad Sci U S A* (1980) 77:3455–9. doi:10.1073/pnas.77.6.3455
18. Vitti P, Valente WA, Ambesi-Impiombato FS, Fenzi GF, Pinchera A, Kohn LD. Graves' IgG stimulation of continuously cultured rat thyroid cells: a sensitive and potentially useful clinical assay. *J Endocrinol Invest* (1982) 5:179–82. doi:10.1007/BF03349476
19. Vitti P, Rotella CM, Valente WA, Cohen J, Aloj SM, Laccetti P, et al. Characterization of the optimal stimulatory effects of Graves' monoclonal and serum Immunoglobulin G on adenosine 3′,5′-monophosphate production in FRTL-5 thyroid cells: a potential clinical assay. *J Clin Endocrinol Metab* (1983) 57:782–91. doi:10.1210/jcem-57-4-782
20. McKenzie JM, Zakarija M. Clinical review 3. The clinical use of thyrotropin receptor antibody measurements. *J Clin Endocrinol Metab* (1989) 69:1093–6. doi:10.1210/jcem-69-6-1093
21. Morris JC, Hay ID, Nelson RE, Jiang NS. Clinical utility of thyrotropin-receptor antibody assays: comparison of radioreceptor and bioassay methods. *Mayo Clin Proc* (1988) 63:707–17. doi:10.1016/S0025-6196(12)65533-5
22. Kohn LD, Valente WA, Grollman EF, Aloj SM, Vitti P, Inventors; Interthyr Research Foundation Inc., Assignee. *Clinical Determination and/or Quantification of Thyrotropin and a Variety of Thyroid Stimulatory or Inhibitory Factors Performed In Vitro with an Improved Thyroid Cell Line, FRTL-5*. United States patent US 4609622 (1986).
23. Vitti P, Chiovato L, Lopez G, Lombardi A, Santini F, Mammoli C, et al. Measurement of TSAb directly in serum using FRTL-5 cells. *J Endocrinol Invest* (1988) 11:313–7. doi:10.1007/BF03350157
24. Yavin E, Yavin Z, Schneider MD, Kohn LD. Monoclonal antibodies to the thyrotropin receptor: implications for receptor structure and the action of autoantibodies in Graves' disease. *Proc Natl Acad Sci U S A* (1981) 78:3180–4. doi:10.1073/pnas.78.5.3180
25. Valente WA, Vitti P, Yavin Z, Yavin E, Rotella CM, Grollman EF, et al. Monoclonal antibodies to the thyrotropin receptor: stimulating and blocking antibodies derived from the lymphocytes of patients with Graves' disease. *Proc Natl Acad Sci U S A* (1982) 79:6680–4. doi:10.1073/pnas.79.21.6680
26. Valente WA, Yavin Z, Yavin E, Grollman EF, Schneider M, Rotella CM, et al. Monoclonal antibodies to the thyrotropin receptor: the identification of blocking and stimulating antibodies. *J Endocrinol Invest* (1982) 5:293–301. doi:10.1007/BF03350517
27. Orgiazzi J, Williams DE, Chopra IJ, Solomon DH. Human thyroid adenyl cyclase-stimulating activity in immunoglobulin G of patients with Graves' disease. *J Clin Endocrinol Metab* (1976) 42:341–54. doi:10.1210/jcem-42-2-341
28. Irvine WJ, Lamberg BA, Cullen DR, Gordin R. Primary hypothyroidism preceding thyrotoxicosis: a report of 2 cases and a review of the literature. *J Clin Lab Immunol* (1979) 2:349–52.
29. Valente WA, Vitti P, Rotella CM, Vaughan MM, Aloj SM, Grollman EF, et al. Antibodies that promote thyroid growth. A distinct population of thyroid-stimulating autoantibodies. *N Engl J Med* (1983) 309:1028–34. doi:10.1056/NEJM198310273091705
30. Tahara K, Grollman EF, Saji M, Kohn LD. Regulation of prostaglandin synthesis by thyrotropin, insulin or insulin-like growth factor-I, and serum in FRTL-5 rat thyroid cells. *J Biol Chem* (1991) 266:440–8.
31. Kohn LD, Shimura H, Shimura Y, Hidaka A, Giuliani C, Napolitano G, et al. The thyrotropin receptor. *Vitam Horm* (1995) 50:287–384. doi:10.1016/S0083-6729(08)60658-5
32. Di Cerbo A, Di Girolamo M, Guardabasso V, De Filippis V, Corda D. Immunoglobulins from Graves' patients stimulate phospholipase-A2 in FRTL-5 thyroid cells. *J Clin Endocrinol Metab* (1992) 74:585–92. doi:10.1210/jc.74.3.585
33. Di Cerbo A, Di Paola R, Menzaghi C, De Filippis V, Tahara K, Corda D, et al. Graves' immunoglobulins activate phospholipase-A2 by recognizing specific epitopes on thyrotropin receptor. *J Clin Endocrinol Metab* (1999) 84:3283–92. doi:10.1210/jcem.84.9.5967
34. Morshed SA, Latif R, Davies TF. Characterization of thyrotropin receptor antibody-induced signaling cascades. *Endocrinology* (2009) 150:519–29. doi:10.1210/en.2008-0878
35. Morshed SA, Ando T, Latif R, Davies TF. Neutral antibodies to the TSH receptor are present in Graves' disease and regulate selective signaling cascades. *Endocrinology* (2010) 151:5537–49. doi:10.1210/en.2010-0424
36. Ihara Y, Kanda Y, Seo M, Watanabe Y, Akamizu T, Tanaka Y. Growth stimulating antibody, as another predisposing factor of Graves' disease (GD): analysis using monoclonal TSH receptor antibodies derived from patients with GD. *Endocr J* (2012) 59:571–7. doi:10.1507/endocrj.EJ11-0348
37. Parmentier M, Libert F, Maenhaut C, Lefort Gerard C, Perret J, Van Sande J, et al. Molecular cloning of the thyrotropin receptor. *Science* (1989) 246:1620–2. doi:10.1126/science.2556796
38. Nagayama Y, Kaufman KD, Seto P, Rapoport B. Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. *Biochem Biophys Res Commun* (1989) 165:1184–90. doi:10.1016/0006-291X(89)92727-7
39. Akamizu T, Ikuyama S, Saji M, Kosugi S, Kozak C, McBride OW, et al. Cloning, chromosomal assignment, and regulation of the rat thyrotropin receptor: expression of the gene is regulated by thyrotropin, agents that increase cAMP levels, and thyroid autoantibodies. *Proc Natl Acad Sci U S A* (1990) 87:5677–81. doi:10.1073/pnas.87.15.5677
40. Ludgate M, Perret J, Parmentier M, Gerard C, Libert F, Dumont JE, et al. Use of recombinant human thyrotropin receptor (TSH-R) expressed in mammalian cell lines to assay TSH-R autoantibodies. *Mol Cell Endocrinol* (1991) 73:R13–8. doi:10.1016/0303-7207(90)90050-I
41. Filetti S, Foti D, Costante G, Rapoport B. Recombinant human thyrotropin (TSH) receptor in a radioreceptor assay for the measurement of TSH receptor autoantibodies. *J Clin Endocrinol Metab* (1991) 72:1096–101. doi:10.1210/jcem-72-5-1096
42. Vitti P, Elisei R, Tonacchera M, Chiovato L, Mancusi F, Rago T, et al. Detection of thyroid-stimulating antibody using Chinese hamster ovary cells transfected

with cloned human thyrotropin receptor. *J Clin Endocrinol Metab* (1993) 76:499–503. doi:10.1210/jcem.76.2.8094393
43. Kim MR, Faiman C, Hoogwerf BJ, Gupta MK. Thyroid-stimulating antibody assay using a human thyrotropin receptor transfected cell line: relationship to clinical features of Graves' disease. *Endocr Pract* (1997) 3:337–43. doi:10.4158/EP.3.6.337
44. Kohn LD, Giuliani C, Montani V, Napolitano G, Ohmori M, Ohta M, et al. Antireceptor immunity. In: Rayner DC, Champion BR, editors. *Thyroid Autoimmunity*. Austin, TX: RG Landes Company (1995). p. 115–70.
45. Rapoport B, Chazenbalk GD, Jaume JC, McLachlan SM. The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. *Endocr Rev* (1998) 19:673–716. doi:10.1210/er.19.6.673
46. Tahara K, Ban T, Minegishi T, Kohn LD. Immunoglobulins from Graves' disease patients interact with different sites on TSH receptor/LH-CG receptor chimeras than either TSH or immunoglobulins from idiopathic myxedema patients. *Biochem Biophys Res Commun* (1991) 179:70–7. doi:10.1016/0006-291X(91)91335-A
47. Tahara K, Ishikawa N, Yamamoto K, Hirai A, Ito K, Tamura Y, et al. Epitopes for thyroid stimulating and blocking autoantibodies on the extracellular domain of the human thyrotropin receptor. *Thyroid* (1997) 7:867–77. doi:10.1089/thy.1997.7.867
48. Nagayama Y, Wadsworth HL, Russo D, Chazenbalk GD, Rapoport B. Binding domains of stimulatory and inhibitory thyrotropin (TSH) receptor autoantibodies determined with chimeric TSH-lutropin/chorionic gonadotropin receptors. *J Clin Invest* (1991) 88:336–40. doi:10.1172/JCI115297
49. Nunez Miguel R, Sanders J, Sanders P, Young S, Clark J, Kabelis K, et al. Similarities and differences in interactions of thyroid stimulating and blocking autoantibodies with the TSH receptor. *J Mol Endocrinol* (2012) 49:137–51. doi:10.1530/JME-12-0040
50. Latif R, Teixeira A, Michalek K, Ali MR, Schlesinger M, Baliram R, et al. Antibody protection reveals extended epitopes on the human TSH receptor. *PLoS One* (2012) 7(6):e44669. doi:10.1371/journal.pone.0044669
51. Davies TF, Latif R. Targeting the thyroid-stimulating hormone receptor with small molecule ligands and antibodies. *Expert Opin Ther Targets* (2015) 19:835–47. doi:10.1517/14728222.2015.1018181
52. Kim WB, Cho BY, Park HY, Lee HK, Kohn LD, Tahara K, et al. Epitopes for thyroid-stimulating antibodies in Graves' sera: a possible link of heterogeneity to differences in response to antithyroid drug treatment. *J Clin Endocrinol Metab* (1996) 81:1758–67. doi:10.1210/jc.81.5.1758
53. Kim WB, Chung HK, Lee HK, Kohn LD, Tahara K, Cho BY. Changes in epitopes for thyroid-stimulating antibodies in Graves' disease sera during treatment of hyperthyroidism: therapeutic implications. *J Clin Endocrinol Metab* (1997) 82:1953–9. doi:10.1210/jc.82.6.1953
54. Kim TY, Park YJ, Park DJ, Chung HK, Kim WB, Kohn LD, et al. Epitopes heterogeneity of thyroid-stimulating antibodies predicts long-term outcome in Graves' patients treated with antithyroid drugs. *J Clin Endocrinol Metab* (2003) 88:117–24. doi:10.1210/jc.2002-020389
55. Costagliola S, Morgenthaler NG, Hoermann R, Badenhoop K, Struck J, Freitag D, et al. Second generation assay for thyrotropin receptor antibodies has superior diagnostic sensitivity for Graves' disease. *J Clin Endocrinol Metab* (1999) 84:90–7. doi:10.1210/jc.84.1.90
56. Sanders J, Oda Y, Roberts S, Kiddie A, Richards T, Bolton J, et al. The interaction of TSH receptor autoantibodies with ^{125}I-labelled TSH receptor. *J Clin Endocrinol Metab* (1999) 84:3797–802. doi:10.1210/jcem.84.10.6071
57. Kamijo K. TSH-receptor antibody measurement in patients with various thyrotoxicosis and Hashimoto's thyroiditis: a comparison of two two-step assays, coated plate ELISA using porcine TSH-receptor and coated tube radioassay using human recombinant TSH-receptor. *Endocr J* (2003) 50:113–6. doi:10.1507/endocrj.50.113
58. Barbesino G, Tomer Y. Clinical utility of TSH receptor antibodies. *J Clin Endocrinol Metab* (2013) 98:2247–55. doi:10.1210/jc.2012-4309
59. Ando T, Latif R, Pritsker A, Moran T, Nagayama Y, Davies TF. A monoclonal thyroid-stimulating antibody. *J Clin Invest* (2002) 110:1667–74. doi:10.1172/JCI0216991
60. Costagliola S, Franssen JDF, Bonomi M, Urizar E, Willnich M, Bergmann A, et al. Generation of a mouse monoclonal TSH receptor antibody with stimulating activity. *Biochem Biophys Res Commun* (2002) 299:891–6. doi:10.1016/S0006-291X(02)02762-6
61. Sanders J, Jeffreys J, Depraetere H, Richards T, Evans M, Kiddie A, et al. Thyroid-stimulating monoclonal antibodies. *Thyroid* (2002) 12:1043–50. doi:10.1089/105072502321085135
62. Ando T, Latif R, Daniel S, Eguchi K, Davies TF. Dissecting linear and conformational epitopes on the native thyrotropin receptor. *Endocrinology* (2004) 145:5185–93. doi:10.1210/en.2004-0789
63. Latif R, Ando T, Davies TF. Monomerization as a prerequisite for intramolecular cleavage and shedding of the thyrotropin receptor. *Endocrinology* (2004) 145:5580–8. doi:10.1210/en.2004-0797
64. Ando T, Davies TF. Monoclonal antibodies to the thyrotropin receptor. *Clin Dev Immunol* (2005) 12:137–43. doi:10.1080/17402520500078238
65. Ando T, Latif R, Davies TF. Antibody-induced modulation of TSH receptor post-translational processing. *J Endocrinol* (2007) 195:179–86. doi:10.1677/JOE-07-0058
66. Sanders J, Evans M, Premawardhana LDKE, Depraetere H, Jeffreys J, Richards T, et al. Human monoclonal stimulating autoantibody. *Lancet* (2003) 362:126–8. doi:10.1016/S0140-6736(03)13866-4
67. Rees Smith B, Bolton J, Young S, Collyer A, Weeden A, Bradbury J, et al. A new assay for thyrotropin receptor autoantibodies. *Thyroid* (2004) 14:830–5. doi:10.1089/1050725042451248
68. Zophel K, Roggenbuck D, von Landenberg P, Wunderlich G, Gruning T, Kotzerke J, et al. TSH receptor antibody (TRAb) assays based on the human monoclonal autoantibody M22 are more sensitive than bovine TSH based assays. *Horm Metab Res* (2010) 42:65–9. doi:10.1055/s-0029-1241196
69. Furmaniak J, Sanders J, Nunez Miguel R, Rees Smith B. Mechanisms of action of TSHR autoantibodies. *Horm Metab Res* (2015) 47:735–52. doi:10.1055/s-0035-1559648
70. Lytton SD, Kahaly GJ. Bioassays for TSH-receptor autoantibodies: an update. *Autoimmun Rev* (2010) 10:116–22. doi:10.1016/j.autrev.2010.08.018
71. Chanzenbalk GD, Pichurin P, Chen CR, Latrofa F, Johnstone AP, McLachlan SM, et al. Thyroid-stimulating antibodies in Graves' diseases preferentially recognize the free subunit, not the holoreceptor. *J Clin Invest* (2002) 110:209–17. doi:10.1172/JCI0215745
72. Chen CR, Pichurin P, Nagayama Y, Latrofa F, Rapoport B, McLachlan SM. The thyrotropin receptor autoantigen in Graves is the culprit as well as the victim. *J Clin Invest* (2003) 111:1897–904. doi:10.1172/JCI200317069
73. Mizutori Y, Chen CR, Latrofa F, McLachlan SM, Rapoport B. Evidence that shed TSH receptor A-subunits drive affinity maturation of autoantibodies causing Graves' disease. *J Clin Endocrinol Metab* (2009) 94:927–35. doi:10.1210/jc.2008-2134
74. Rapoport B, McLachlan SM. TSH receptor cleavage into subunits and shedding of the A-subunit; a molecular and clinical perspective. *Endocr Rev* (2016) 37:113–34. doi:10.1210/er.2015-1098
75. Watson PF, Ajjan RA, Phipps J, Metcalfe R, Weetman AP. A new chemiluminescent assay for the rapid detection of thyroid stimulating antibodies in Graves' disease. *Clin Endocrinol* (1998) 49:577–81. doi:10.1046/j.1365-2265.1998.00619.x
76. Giuliani C, Cerrone D, Harii N, Thornton M, Kohn LD, Dagia NM, et al. A TSHr-LH/CGr chimera that measures functional TSAb in Graves' disease. *J Clin Endocrinol Metab* (2012) 97:E1106–15. doi:10.1210/jc.2011-2893
77. Giuliani C, Cerrone D, Harii N, Thornton M, Kohn LD, Dagia NM, et al. A TSHr-LH/CGr chimera that measures functional thyroid-stimulating autoantibodies (TSAb) can predict remission or recurrence in Graves' patients undergoing antithyroid drug (ATD) treatment. *J Clin Endocrinol Metab* (2012) 97:E1080–7. doi:10.1210/jc.2011-2897
78. Hwang S, Shin DY, Song MK, Lee EJ. High cut-off value of a chimeric TSH receptor (Mc4)-based bioassay may improve prediction of relapse in Graves' disease for 12 months. *Endocrine* (2015) 48:89–95. doi:10.1007/s12020-014-0325-8
79. Lytton SD, Ponto KA, Kanitz M, Matheis N, Kohn LD, Kahaly GJ. A novel thyroid stimulating immunoglobulin bioassay is a functional indicator of activity and severity of Graves' orbitopathy. *J Clin Endocrinol Metab* (2010) 95:2123–31. doi:10.1210/jc.2009-2470
80. Jang SY, Shin DY, Lee EJ, Yoon JS. Clinical characteristics of Graves' orbitopathy in patients showing discrepancy between levels from TBII assay and TSI bioassay. *Clin Endocrinol* (2014) 80:591–7. doi:10.1111/cen.12318
81. Diana T, Brown RS, Bossowski A, Segni M, Niedziela M, Koning J, et al. Clinical relevance of thyroid-stimulating autoantibodies in pediatric Graves'

disease. A multicenter study. *J Clin Endocrinol Metab* (2014) 99:1648–55. doi:10.1210/jc.2013-4026

82. Ponto KA, Kanitz M, Olivo PD, Pitz S, Pfeiffer N, Kahaly GJ. Clinical relevance of thyroid-stimulating immunoglobulins in Graves' ophthalmopathy. *Ophthalmology* (2011) 118:2279–85. doi:10.1016/j.ophtha.2011.03.030
83. Kamijo K, Murayama H, Uzu T, Togashi K, Kahaly GJ. A novel bioreporter assay for thyrotropin receptor antibodies using a chimeric thyrotropin receptor (Mc4) is more useful in differentiation of Graves' disease from painless thyroiditis than conventional thyrotropin-stimulating antibody assay using porcine thyroid cells. *Thyroid* (2010) 20:851–6. doi:10.1089/thy.2010.0059
84. Diana T, Kanitz M, Lehman M, Li Y, Olivo PD, Kahaly GJ. Standardization of a bioassay for thyrotropin receptor stimulating autoantibodies. *Thyroid* (2015) 25:169–75. doi:10.1089/thy.2014.0346
85. Kohn LD, Brown J, Scholl D, Li Y, Napolitano G, Inventors; Diagnostic Hybrids Inc., Assignee. *Sensitive and Rapid Methods of Using Chimeric Receptors to Identify Autoimmune Disease and Assess Disease Severity.* United States patent US 8563257B2 (2013).
86. Frank CU, Braeth S, Dietrich JW, Wanjura D, Loos U. Bridge technology with TSH receptor chimera for sensitive direct detection of TSH receptor antibodies causing Graves' disease: analytical and clinical evaluation. *Horm Metab Res* (2015) 47:880–8. doi:10.1055/s-0035-1554662

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer TA and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

frontiers in Endocrinology

GENERAL COMMENTARY
published: 15 February 2017
doi: 10.3389/fendo.2017.00028

TSH Receptor Antibody Functionality and Nomenclature

George J. Kahaly and Tanja Diana*

Molecular Thyroid Research Laboratory, Department of Medicine I, Johannes Gutenberg University Medical Center, Mainz, Germany

Keywords: Graves' disease, functional TSH receptor antibodies, stimulating antibodies, blocking antibodies, nomenclature

A commentary on

Graves' disease
by Smith TJ, Hegedus LN. Engl J Med (2016) 375(16):1552–65. doi:10.1056/NEJMra1510030

This general commentary on the above, recently published *New England Journal of Medicine* review article wishes to clarify both the nomenclature as well as the role of autoantibodies (Ab) to the TSH receptor (TSH-R) pertaining to the serological diagnosis of Graves' disease (GD).

Various terms have been used to describe the different types of TSH-R-Ab. It is important for the clinician to be aware of the different nomenclature as this will frequently reflect which assay is performed by the laboratory (**Table 1**). TSH-R-Ab, often referred to as TRAb, refers to any type of Ab interacting specifically with the TSH-R. Because these Ab are commonly assessed in a competitive binding assay, they are referred to as TSH-R-binding inhibitory immunoglobulins (TBII). By contrast, cell-based bioassays measure either TSH-R stimulatory antibodies (TSAb) or TSH-R-stimulating immunoglobulins, or alternately TSH-R-blocking antibodies (TBAb) or TSH-R-blocking immunoglobulins. Alternative terminologies for blocking antibodies are TSH-R-stimulating blocking Ab or TSH-R-blocking Ab (TRBAb). In this commentary, we will use TSH-R-Ab as a general term to refer to anti-TSH-R-Ab irrespective of the specific assay used. We will use TBII to refer to the Ab measured *via* binding assays, whereas Ab measured *via* bioassays will be referred to as TSAb for stimulatory and TBAb for blocking Ab.

Graves' disease is caused by persistent, unregulated stimulation of thyroid cells by TSH-R-stimulating Ab (TSAb) that activate the TSH-R (1). TSAb, like TSH, bind primarily to the large amino terminal ectodomain of the TSH-R and activate the cAMP signal transduction pathway leading to stimulation of thyroid hormone production and proliferation of thyrocytes. Since the discovery of TSAb as the causative agent of GD, there have been numerous studies that have demonstrated the significance of the levels of these Ab during the course of the disease as well as during antithyroid drug treatment in both adults and children (2, 3). Other types of TSH-R antibodies can antagonize or block the action of TSH and in doing so cause hypothyroidism in certain patients with various types of autoimmune thyroiditis, particularly Hashimoto's thyroiditis. TSH-R antibodies that neither induce the cAMP signal pathway nor block the binding of TSH are referred to as neutral or recently "cleavage" Ab and currently are not known to have a functional effect (4). There is evidence, however, that neutral Ab may induce signaling pathways distinct from the cAMP pathway and may induce apoptosis (5).

As strongly recommended in the recently published hyperthyroidism guidelines of the American Thyroid Association (6), measurement of TSH-R-Ab is indicated both for the accurate and early diagnosis of autoimmune induced hyperthyroidism as well as during the management of patients with GD. Functional TSH-R-stimulating antibodies (TSAb) are causative of both the hyperthyroidism and the extra thyroidal manifestations of GD (7). TSAb can be sensitively and exclusively measured

OPEN ACCESS

Edited by:
Cesidio Giuliani,
University of Chieti-Pescara, Italy

Reviewed by:
Alessandro Antonelli,
University of Pisa, Italy

****Correspondence:***
George J. Kahaly
gkahaly@uni-mainz.de

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *10 January 2017*
Accepted: *01 February 2017*
Published: *15 February 2017*

Citation:
Kahaly GJ and Diana T (2017) TSH Receptor Antibody Functionality and Nomenclature. Front. Endocrinol. 8:28. doi: 10.3389/fendo.2017.00028

TABLE 1 | Terminology for TSH receptor antibodies used in bioassays and binding assays.

	Abbreviation
Cell-based bioassay	
• TSH-R-stimulating antibodies	TSAb
• TSH-R-stimulating immunoglobulins	TSI
• TSH-R-blocking antibodies	TBAb, TSB-Ab, or TRBAb
• TSH-R-stimulating blocking antibodies	TRBAb
• TSH-R-blocking immunoglobulins	TBI
Competitive-binding assay	
• TSH-R-binding inhibitory immunoglobulins	TBII

with validated bioassays that are available worldwide (8–11). In particular, the analytical performance and clinical utility of a FDA-cleared, stimulatory TSH-R bioassay in a large collective of patients with GD, both prior to as well as during medical antithyroid treatment, has been shown (12). In addition, a multicenter trial involving seven American and European academic referral centers confirmed the very high specificity, sensitivity, and positive and negative predictive values of this tool for the diagnosis of GD in children (13). Standardization and calibration of this bioassay, using a purely stimulatory human monoclonal TSH-R-Ab as international standard, allowed results to be reported in international units per liter (14). This has facilitated comparison of bioassay results with commercially available automated TSH-R-binding or TBII assays. A recent comparative study of seven immunoassays has shown that bioassays for TSH-R-Ab are more sensitive than the automated binding assays and exclusively differentiate between stimulatory and blocking Ab activity (15). Also, TSAb are a highly sensitive and predictive biomarker of the extra thyroidal manifestations of GD (16–18). Furthermore, the clinical relevance of the measurement of TSH-R-Ab and of TSAb in particular, during pregnancy in patients with autoimmune thyroid disease, was recently documented in a newborn with fetal/neonatal autoimmune thyrotoxicosis (19). Finally, incorporation and early utilization of TSAb into current diagnostic algorithms was shown to confer a 46% shortened time to diagnosis of GD and a cost savings of 47% (20).

AUTHOR CONTRIBUTIONS

The two authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

1. Smith TJ, Hegedus L. Graves' disease. *N Engl J Med* (2016) 375(16):1552–65. doi:10.1056/NEJMra1510030
2. Botero D, Brown RS. Bioassay of thyrotropin receptor antibodies with Chinese hamster ovary cells transfected with recombinant human thyrotropin receptor: clinical utility in children and adolescents with Graves disease. *J Pediatr* (1998) 132(4):612–8.
3. Smith J, Brown RS. Persistence of thyrotropin (TSH) receptor antibodies in children and adolescents with Graves' disease treated using antithyroid medication. *Thyroid* (2007) 17(11):1103–7. doi:10.1089/thy.2007.0072
4. Morshed SA, Ando T, Latif R, Davies TF. Neutral antibodies to the TSH receptor are present in Graves' disease and regulate selective signaling cascades. *Endocrinology* (2010) 151(11):5537–49. doi:10.1210/en.2010-0424
5. Morshed SA, Ma R, Latif R, Davies TF. How one TSH receptor antibody induces thyrocyte proliferation while another induces apoptosis. *J Autoimmun* (2013) 47:17–24. doi:10.1016/j.jaut.2013.07.009
6. Ross DS, Burch HB, Cooper DS, Greenlee MC, Laurberg P, Maia AL, et al. 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. *Thyroid* (2016) 26(10):1343–421. doi:10.1089/thy.2016.0229
7. Bahn RS. Graves' ophthalmopathy. *N Engl J Med* (2010) 362(8):726–38. doi:10.1056/NEJMra0905750
8. Lytton SD, Kahaly GJ. Bioassays for TSH-receptor autoantibodies: an update. *Autoimmun Rev* (2010) 10(2):116–22. doi:10.1016/j.autrev.2010.08.018
9. Kahaly GJ. Bioassays for TSH receptor antibodies: quo vadis? *Eur Thyroid J* (2015) 4(1):3–5. doi:10.1159/000375445
10. FIRS Laboratories, RSR Ltd. *Assay Service. Thyroid Stimulating Autoantibody Bioassay*. Available from: http://www.rsrltd.com/assay_service.html (accessed September 20, 2013).
11. Araki N, Iida M, Amino N, Morita S, Ide A, Nishihara E, et al. Rapid bioassay for detection of thyroid-stimulating antibodies using cyclic adenosine monophosphate-gated calcium channel and aequorin. *Eur Thyroid J* (2015) 4(1):14–9. doi:10.1159/000371740
12. Leschik JJ, Diana T, Olivo PD, Konig J, Krahn U, Li Y, et al. Analytical performance and clinical utility of a bioassay for thyroid-stimulating immunoglobulins. *Am J Clin Pathol* (2013) 139(2):192–200. doi:10.1309/AJCPZUT7CNUEU7OP
13. Diana T, Brown RS, Bossowski A, Segni M, Niedziela M, Konig J, et al. Clinical relevance of thyroid-stimulating autoantibodies in pediatric Graves' disease – a multicenter study. *J Clin Endocrinol Metab* (2014) 99(5):1648–55. doi:10.1210/jc.2013-4026
14. Diana T, Kanitz M, Lehmann M, Li Y, Olivo PD, Kahaly GJ. Standardization of a bioassay for thyrotropin receptor stimulating autoantibodies. *Thyroid* (2015) 25(2):169–75. doi:10.1089/thy.2014.0346
15. Diana T, Wuster C, Kanitz M, Kahaly GJ. Highly variable sensitivity of five binding and two bio-assays for TSH-receptor antibodies. *J Endocrinol Invest* (2016) 39(10):1159–65. doi:10.1007/s40618-016-0478-9
16. Lytton SD, Ponto KA, Kanitz M, Matheis N, Kohn LD, Kahaly GJ. A novel thyroid stimulating immunoglobulin bioassay is a functional indicator of activity and severity of Graves' orbitopathy. *J Clin Endocrinol Metab* (2010) 95(5):2123–31. doi:10.1210/jc.2009-2470
17. Ponto KA, Kanitz M, Olivo PD, Pitz S, Pfeiffer N, Kahaly GJ. Clinical relevance of thyroid-stimulating immunoglobulins in Graves' ophthalmopathy. *Ophthalmology* (2011) 118(11):2279–85. doi:10.1016/j.ophtha.2011.03.030
18. Kahaly GJ, Diana T, Glang J, Kanitz M, Pitz S, Konig J. Thyroid stimulating antibodies are highly prevalent in Hashimoto's thyroiditis and associated orbitopathy. *J Clin Endocrinol Metab* (2016) 101(5):1998–2004. doi:10.1210/jc.2016-1220
19. Kiefer FW, Klebermass-Schrehof K, Steiner M, Worda C, Kasprian G, Diana T, et al. Fetal/neonatal thyrotoxicosis in a newborn from a hypothyroid woman with Hashimoto's thyroiditis. *J Clin Endocrinol Metab* (2017) 102(1):6–9. doi:10.1210/jc.2016-2999
20. McKee A, Peyerl F. TSI assay utilization: impact on costs of Graves' hyperthyroidism diagnosis. *Am J Manag Care* (2012) 18(1):e1–14.

Conflict of Interest Statement: TD has nothing to disclose. GK consults for Quidel, USA.

frontiers in Endocrinology

REVIEW
published: 30 June 2017
doi: 10.3389/fendo.2017.00137

Thyroid-Stimulating Hormone Receptor Antibodies in Pregnancy: Clinical Relevance

Ines Bucci, Cesidio Giuliani and Giorgio Napolitano*

Unit of Endocrinology, Department of Medicine and Sciences of Aging, Ce.S.I.-Me.T., University of Chieti-Pescara, Chieti, Italy

Graves' disease is the most common cause of thyrotoxicosis in women of childbearing age. Approximately 1% of pregnant women been treated before, or are being treated during pregnancy for Graves' hyperthyroidism. In pregnancy, as in not pregnant state, thyroid-stimulating hormone (TSH) receptor (TSHR) antibodies (TRAbs) are the pathogenetic hallmark of Graves' disease. TRAbs are heterogeneous for molecular and functional properties and are subdivided into activating (TSAbs), blocking (TBAbs), or neutral (N-TRAbs) depending on their effect on TSHR. The typical clinical features of Graves' disease (goiter, hyperthyroidism, ophthalmopathy, dermopathy) occur when TSAbs predominate. Graves' disease shows some peculiarities in pregnancy. The TRAbs disturb the maternal as well as the fetal thyroid function given their ability to cross the placental barrier. The pregnancy-related immunosuppression reduces the levels of TRAbs in most cases although they persist in women with active disease as well as in women who received definitive therapy (radioiodine or surgery) before pregnancy. Changes of functional properties from stimulating to blocking the TSHR could occur during gestation. Drug therapy is the treatment of choice for hyperthyroidism during gestation. Antithyroid drugs also cross the placenta and therefore decrease both the maternal and the fetal thyroid hormone production. The management of Graves' disease in pregnancy should be aimed at maintaining euthyroidism in the mother as well as in the fetus. Maternal and fetal thyroid dysfunction (hyperthyroidism as well as hypothyroidism) are in fact associated with several morbidities. Monitoring of the maternal thyroid function, TRAbs measurement, and fetal surveillance are the mainstay for the management of Graves' disease in pregnancy. This review summarizes the biochemical, immunological, and therapeutic aspects of Graves' disease in pregnancy focusing on the role of the TRAbs in maternal and fetal function.

Keywords: thyroid-stimulating hormone receptor antibodies, Graves' disease, pregnancy, fetal hyperthyroidism, neonatal hyperthyroidism

OPEN ACCESS

Edited by:
Alessandro Antonelli, University of Pisa, Italy

Reviewed by:
Enke Baldini, Sapienza Università di Roma, Italy
Roberto Vita, University of Messina, Italy

****Correspondence:***
Ines Bucci
ibucci@unich.it

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *02 February 2017*
Accepted: *02 June 2017*
Published: *30 June 2017*

Citation:
Bucci I, Giuliani C and Napolitano G (2017) Thyroid-Stimulating Hormone Receptor Antibodies in Pregnancy: Clinical Relevance. Front. Endocrinol. 8:137. doi: 10.3389/fendo.2017.00137

INTRODUCTION

Pregnancy represents a challenge to the maternal thyroid gland: the various hormonal variations and the increased metabolic demands occurring during gestation deeply affect thyroid function. This means that several changes in the thyroid hormone production and metabolism are expected during gestation (1). During the first trimester, the human chorionic gonadotropin (HCG) hormone, which

shares some structural homologies with thyroid-stimulating hormone (TSH), acts as a thyrotropic agonist, overriding the normal action of the hypothalamic–pituitary–thyroid feedback system. The result of this is a transient increase in free thyroxine (FT4) and a transient reduction in TSH whose plasma concentrations are inversely related to those of the HCG. Due to the high levels of estrogens, the serum thyroxine-binding globulin (TBG) concentration rises almost twofold during the first 20 weeks of gestation and remains at a high level until delivery. This means both serum total thyroxine (T4) and triiodothyronine (T3) concentrations increase while their respective free fractions (FT4, FT3) decrease as low as 10–15%. As a consequence, the pituitary increase secretion of TSH whose concentrations, following the first trimester, return steadily to the normal range and show a slight trend toward an increase in response to the decreased serum-free thyroid hormone levels (2). A new equilibrium is reached with an increase in thyroid hormone production of approximately 50% by the maternal thyroid. In order to achieve greater thyroid hormone production, a higher iodine intake is needed in pregnant women due to a pregnancy-related increase in renal excretion and fetal iodine requirement (3). Pregnancy-related changes in thyroid physiology lead to changes in the thyroid function tests, and therefore, parameters of healthy pregnant women differ from those of euthyroid non-pregnant women. The trimester specific range for TSH, as defined in populations with optimal iodine intake, need to be applied while the interpretation of FT4 values necessitates trimester and method-specific ranges given a significant method-dependent variation in the FT4 measurement in pregnancy (4, 5). In summary, the maternal thyroid gland is designed to increase the thyroid hormone secretion and this could be achieved when the gland is both anatomically and functionally intact as well as the iodine intake being at an adequate level (2). Maternal thyroid hormones play an important role in fetal brain development and because the fetal thyroid produces thyroid hormone starting from week 10–12 of gestation and complete maturation of the hypothalamic–pituitary–thyroid axis is reached at week 20, the fetal development depends on the maternal thyroid for the first half of a pregnancy (6). Thyroid diseases are common in pregnancy and uncontrolled thyroid dysfunction (both overt hypothyroidism and overt hyperthyroidism) is associated with infertility, pregnancy loss, and maternal and fetal/neonatal complications (7). Consequently, the diagnosis and management of thyroid disease in women during preconception, pregnancy, and the postpartum (PP) period is the subject of major attention of scientific associations. Several guidelines have been published and very recently updated (8, 9). Most thyroid diseases affecting childbearing women are autoimmune and up to 20% of pregnant women screened during the first trimester of gestation had positive thyroid autoantibodies (10). Thyroid autoimmunity is associated with infertility as well as with different pregnancy complications such as miscarriage, preterm delivery, and PP depression (11, 12). Among autoimmune thyroid diseases, Graves' disease is of particular relevance in pregnancy. In fact, Graves' disease together with its therapy could affect maternal and fetal outcome; however, pregnancy by itself could change the presentation and course of Graves' disease. This review is focused on the role of TSH receptor antibodies (TRAbs) that represent the hallmark of Graves' disease and are able to influence, contemporarily and/or independently, both the maternal and the fetal thyroid function.

GRAVES' DISEASE IN PREGNANCY

Graves' disease occurs before pregnancy in 0.4–1% of women and in 0.2–0.4% during pregnancy, representing the most common cause (85%) of either overt or subclinical hyperthyroidism in women of reproductive age (11, 13). A more frequent and peculiar form of hyperthyroidism in pregnancy is the gestational transient thyrotoxicosis (GTT) whose prevalence in Europe is estimated between 2 and 3% with higher levels (5.5–11%) in Asia (2, 14, 15). GTT is defined as transient thyrotoxicosis caused by the stimulating effect the β-HCG has on the TSH receptor toward the end of the first trimester of gestation and is frequently associated with hyperemesis gravidarum and twin pregnancies. The prevalence of other causes of thyrotoxicosis in pregnancy (multinodular toxic goiter, toxic adenoma, subacute or silent thyroiditis, iodide-induced thyrotoxicosis, thyrotoxicosis factitia, hydatidiform mole, and hyperplacentosis) is negligible (2). In a population-based study in Denmark, which included 403,958 women, the incidence of hyperthyroidism (defined by redeemed prescription of antithyroid drugs (ATDs) and assumed to be Graves' disease) was high early in pregnancy, declined during gestation and significantly increased at 7–9 months PP. Such a pattern was not observed for other autoimmune diseases (16). This observation acts as a clue as to the peculiar course of Graves' disease in pregnancy. Different clinical scenarios can be observed in pregnant women: (1) stable active diseases receiving ATDs, (2) relapse in pregnancy after a ATDs course-induced remission, (3) *de novo* onset early in pregnancy, and (4) previous surgery or radioiodine treatment with persistence of TRAbs (**Table 1**). Women with a stable disease on ATDs could experience the worsening of hyperthyroidism early in pregnancy due to the additive thyroid-stimulating effect of HCG (**Figure 1**). In the same way, undiagnosed, subclinical Graves' hyperthyroidism may become overt early in pregnancy. Early pregnancy relapse after ATDs withdrawal can be observed in women who have been treated for less than 6 months, or have ophthalmopathy or high levels of TRAbs (17). Later in pregnancy, Graves' hyperthyroidism improves with remission in up to 30% of women by the middle of the third trimester and with relapse during the PP period (18). The main explanation for this course is the decrease of TRAbs due to the pregnancy-related immunosuppression/hemodilution. A contribution to the clinical improvement is the reduction of iodine pool and the increased binding capacity of TBG resulting in reduced free and active thyroid hormones.

Diagnosis, Complications, and Therapy

The diagnosis of hyperthyroidism in pregnancy is straightforward in women with Graves' disease already known before pregnancy

TABLE 1 | Clinical scenarios of Graves' disease in pregnancy.

Stable Graves' disease receiving antithyroid drugs (ATDs)
Relapsed Graves' disease after an ATDs course
De novo onset of Graves' disease in early pregnancy
History of Graves' disease treated with radioiodine/surgery

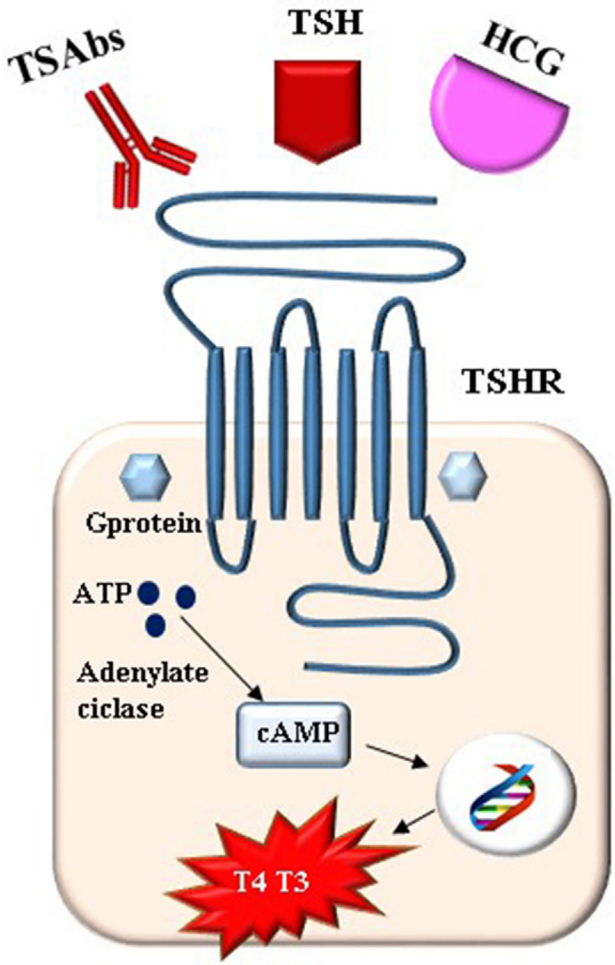


FIGURE 1 | The stimulation of TSH receptor (TSHR) in pregnancy.

since these women undergo pre-pregnancy counseling and monitoring at early stages. If this is not the case, the main diagnostic challenge is to identify signs and symptoms of thyrotoxicosis as well as to distinguish Graves' disease from GTT. The symptoms vary depending on the degree of thyrotoxicosis. The clinical manifestations are often well pronounced in Graves' disease while they may be absent or hidden in GTT. Some symptoms and signs may overlap with those typical of a hyperdynamic state of pregnancy, while others are more specific such as tachycardia, frequently exceeding 100 bpm, proximal muscle weakness and the failure to gain weight despite an increased appetite. The presence of goiter and/or of extra thyroidal manifestations, such as ophthalmopathy or dermopathy, is a clinical clue for Graves' disease (2, 17). Clinical suspicion of hyperthyroidism needs to be confirmed by the finding of elevated serum FT4 concentrations and of suppressed serum TSH levels. The measurement of TRAbs is helpful in clarifying the etiology of thyrotoxicosis and a positive result strongly supports the diagnosis of Graves' disease (9). Thyroid ultrasound and color Doppler could add clues showing a hypoechoic pattern and intense vascularity, radionuclide scintigraphy or radioiodine uptake are contraindicated during pregnancy. The measurement of TRAbs could have higher diagnostic value early in pregnancy given that their levels tend to decrease as pregnancy progress due to the physiological immunosuppression (2). The clinical significance of a positive TRAbs result goes beyond the diagnostic role for the mother since it is more relevant to predict fetal thyroid dysfunction. TRAbs cross the placenta and could cause goiter and hyperthyroidism in the fetus; therefore, Graves' disease in pregnancy has long been recognized as an indisputable indication for TRAbs measurement (8, 9, 19). Several prospective and retrospective studies have highlighted that overt hyperthyroidism is associated with several adverse effects on pregnancy outcomes, which are directly related to the duration of thyrotoxicosis throughout a pregnancy. The most frequent complication is pregnancy-induced hypertension; the risk of eclampsia is five times higher in uncontrolled hyperthyroid women compared to controlled women and non-hyperthyroid pregnant women (20, 21). The overlap of hypertension to the left ventricular dysfunction induced by prolonged thyrotoxicosis could precipitate into congestive heart failure. Overt hyperthyroidism also increases the risk for intrauterine growth restriction, spontaneous preterm labor, preterm birth, gestational diabetes mellitus, cesarean delivery, and low birth weight infants. The highest risk for still birth (up to eight times) and low birth weight is observed in women with uncontrolled disease (22, 23). Subclinical hyperthyroidism is well tolerated from the mother and the fetus, while overt hyperthyroidism needs to be adequately treated in order to prevent obstetric and medical complications. The treatment of choice for overt hyperthyroidism in pregnant women is ATDs (8, 9, 13). Radioiodine treatment is contraindicated in pregnancy. Surgery is indicated in selected cases such as severe side effects of ATDs, or uncontrolled thyrotoxicosis despite high ATDs doses, and should be planned preferably in the second trimester of pregnancy to minimize the potential teratogenic effects of anesthetic agents. The drugs used are the propylthiouracil (PTU) and methimazole (MMI). PTU is generally preferred during the first trimester of pregnancy and then changed to MMI because of the risk of MMI-induced embryopathy, mainly aplasia cutis, esophageal, and choanal atresia (24–26) whose prevalence is reported higher than previously thought (27). The prevalence of the PTU-associated, but less severe, birth defects is not negligible (9, 28). After the first trimester, MMI is the preferred ATD because PTU has a greater risk of hepatotoxicity (9). The starting dose varies according to the extent of thyrotoxicosis and the equivalent potency MMI to PTU is 1:20 (9). It is worth remembering that both MMI and PTU cross the placenta (29) and tend to have greater effect on the fetal thyroid function compared to the mother. Therefore, ATD doses need to be tailored to correct the maternal thyrotoxicosis and to avoid fetal hypothyroidism, which is detrimental for the fetal brain development. This is achieved by using the minimum dose of ATDs to maintain the concentration of FT4 in the high values of the normal non-pregnant range irrespective of TSH levels whose normalization would require doses able to determine fetal hypothyroidism. In most cases due to the pregnancy-induced amelioration of Graves' disease, the dose can be gradually reduced and ATDs even discontinued in third trimester especially in women with negative or decreasing TRAbs.

TRAbs IN PREGNANCY

In pregnancy, as in the non-pregnant state, TRAbs are a hallmark of Graves' disease. TSH receptor (TSHR), thyroglobulin (Tg), and thyroid peroxidase (TPO) are the immune targets of autoreactive T cells and autoantibodies in autoimmune thyroid disease, but while Tg and TPO autoantibodies are detected also in healthy subjects, anti-TRAbs can be found only in sera of most patients with Graves' disease and in 10–15% of patients with Hashimoto's thyroiditis (30). TRAbs are also unique among antithyroid autoantibodies having a key pathogenetic role in determining the hyperthyroidism and the extra thyroidal manifestation of Graves' disease such as ophthalmopathy (31). It is worth to note that the term TRAbs means antibodies able to interact the TSH receptor, regardless of their action and of the method employed to detect them (32). The characterization of TRAbs has been the subject of research since the original description of long-acting thyroid stimulators in the fifties (33). Nowadays, it is known that TRAbs are able to influence thyroid function acting on the TSHR in different ways: stimulating (TSAbs), blocking (TBAbs), and without determining functional response with a neutral effect (N-TRAbs). TSAbs are the hallmark of Graves' disease (31). TBAbs can also be observed in Graves' disease being responsible for the evolution toward hypothyroidism in a small percentage of patients while they have a pathogenetic role in the atrophic form of Hashimoto's thyroiditis (34). Switching between TBAb and TSAb (or *vice versa*) occurs, although rarely, in hypothyroid patients and in ATDs treated patients with Graves' disease (35).

TRAbs Assays

Given the pathogenetic and prognostic role of TRAbs in Graves' disease, it is not surprising that research has made a strong effort in the development of methods to quantify and characterize TRAbs, which could be useful in the clinical management. Different assays for the detection of TRAbs have been available for more than 30 years over different generations of laboratory methods and great improvement in sensitivity and specificity have been achieved. The description of the different assay methods is beyond the scope of this review and is exhaustively detailed elsewhere (30, 31, 36–39). Briefly, two different methods can be distinguished: "receptor assays" and "bioassays." Receptor assays measure TSH-binding inhibiting immunoglobulins (TBII) meaning that they detect serum autoantibodies by their capacity to compete for the binding of labeled TSH to an *in vitro* TSH receptor preparation. Three generations of TBII assay have been developed. The first-generation assays are competitive immunoassays in liquid phase and they detect the inhibition, by antibodies in the patient's serum, of the binding of radio- or enzyme-labeled TSH to thyroid membrane extracts. The second generation assays are competitive immunoassays in solid phase, which use recombinant human TSHR or porcine TSHR. The third-generation assays are solid-phase competitive immunoassays based on the competition between antibodies in the patient's serum and a human labeled thyroid-stimulating monoclonal antibody (M22) for the binding to TSHR. Increasing sensitivity and specificity has been achieved through the different generations of immunoassays and great progress has been made in the automation of assays. Overall the sensitivity and specificity of the second- and third-generation TRAbs assays are 86.5 and 97.4%, and 97 and 99.2%, respectively, with little difference between the types of immunoassay methods used (human or porcine receptor, manual or automated procedure) (39). The major limitation of receptor assays is that they are not able to evaluate functional properties of the TRAbs (i.e., they do not differentiate between TSAb and TBAb in serum samples). Therefore, they do not predict the phenotypes of Graves' disease and a lack of correlation between TRAbs levels, measured using these assays, and the clinical and biochemical severity of the disease can be observed. Bioassays are functional tests that have the main advantage to detect the functional properties of TRAbs, i.e., stimulating (TSAbs) or blocking (TBAbs). This is accomplished by incubating the patient's serum with cultured cells natively or artificially expressing TSHR (FRTL-5 or CHO cells) and then measuring the cyclic AMP production by the use of radioimmunoassay or by chemiluminescent assay. Similar to immunoassays, bioassays have gone through significant improvement from technically demanding methods to assays now available as commercial kits (39). A new Mc4 bioassay that measures only TSAbs, without interference of blocking TRAbs, is now available in commercial kit showing good sensitivity and specificity (40, 41). This assay selectively detects TSAbs because it is based on cells expressing a chimeric receptor that, compared to the wild-type, retains the main binding site of the TSAbs, but loses the main epitope recognized by TBAbs, which is replaced with the same receptor portion of the LH/hCG. On the other hand, a bioassay selectively detecting the TBAbs has been developed using a chimeric TSHR (42). Pregnancy entails several differences in interpretation, behavior, and in the role and significance of TRAbs compared to a non-pregnant state. The still point is the fact that during pregnancy, TRAbs readily cross the placenta. Therefore, potential effects on expectant mothers, as well as fetal thyroid function during pregnancy and, again, with neonatal and mother thyroid function in the PP are dealt with (43). Starting from methodological issues of TRAbs detection, the best TRAbs assay used in pregnancy should be the bioassay since the functional activity of the TRAbs is crucial, especially for the fetus (11). In fact, if the TRAbs are detected in a hyperthyroid pregnant women, they obviously have stimulating properties, this is not true in women who show detectable TRAbs levels but are no longer hyperthyroid having received definitive treatment for their disease (i.e., surgery or radioiodine). In these women, information on biological activity of the TRAbs is crucial to predict their effect on the fetus. Receptor assays and bioassays have a complementary role in pregnancy (36, 44).

TRAbs Changes during Pregnancy

Regarding the behavior of the TRAbs, it is remarkable to note that due to the pregnancy-induced immunosuppression autoantibodies levels tend to decrease throughout pregnancy. The most typical scenario is that the TRAbs are detectable in the first trimester, but their levels decrease after 20 weeks of gestation becoming undetectable toward the term of pregnancy. This reflects the amelioration in thyrotoxicosis commonly observed. A study of 45 pregnant GD women (20 treated with ATDs throughout pregnancy and 20 in remission before pregnancy)

showed a significant decrease in the TRAbs levels (measured by a first-generation immunoassay) with a significant rebound PP (45). In a study from Japan, the TRAbs levels were measured serially in 23 women from early to late pregnancy using four methods (first-, second-, and third-generation TBII assays and bioassay) and a decrease in the TRAbs, irrespective of the assay method used, was observed as pregnancy progressed (46). In a more recent study of 42 pregnant women, TRAbs levels (measured by second-generation TBII assay) decreased or remained stable in 86% of patients while rose in 14% (47). Nevertheless Graves' disease's course is variable in pregnancy as well as in a non-pregnant state and therefore there are women whose TRAbs, although at low levels, remain stable in pregnancy as well as women with more severe Graves' disease with high levels of TRAbs not decreasing throughout gestation (**Figure 2**) (48). The disappearance of the TRAbs in pregnant women with Graves' disease, who are euthyroid on a low dose of ATDs, supports the decision to reduce or withdraw medications in late pregnancy. In this scenario, in fact, fetal/neonatal hyperthyroidism is less likely in respect to an ATD-induced fetal hypothyroidism (9). TRAbs could persist for a varied amount of time after definitive therapy for Graves' disease (radioiodine or surgery). It has been established that radioiodine therapy can lead to the worsening of autoimmunity with increasing TRAbs levels (49, 50). In a prospective randomized study, the TRAbs were serially measured in patients treated with ATDs, subtotal thyroidectomy, and radioiodine therapy. During ATDs treatment and after surgery, the TRAbs levels gradually decreased to reach the upper level of the normal reference interval for the assay after approximately 1 year and disappeared in 70–80% of the patients after 18 months. After radioiodine, an increase in the TRAbs was observed immediately after therapy with a maximal value at 3 months. Thereafter, levels slowly returned to pretreatment levels in 1 year, and continued slowly to decrease; however, average values were well above the normal reference throughout the 5 years with approximately 40% of patients still TRAbs-positive (51). In a recent study, a serial evaluation of TRAbs levels, measured by a quantitative third-generation assay, after total thyroidectomy showed that the TRAbs values decreased rapidly in most of the patients, especially within the early postoperative period (3 months). Nevertheless, the TRAbs half-life ranged from 3 months in patients with Graves' disease not complicated with ophthalmopathy and not smoking, to 5 months in patients with ophthalmopathy or smoking and up to 1 year in patients with ophthalmopathy and smoking (52). The course of the TRAbs after surgery and/or radioiodine need to be kept in mind in order to estimate the time needed to achieve the maternal safe value in women planning pregnancy (53). It could be said that the TRAbs could persist beyond the suggested interval of 4–6 months to avoid conception for radioprotection and also beyond the time to reach stable euthyroidism after surgery (8, 9). As mentioned above, this is the only clinical situation where the bioactivity of the TRAbs may need to be known since their effects on the fetus cannot be predicted from the maternal thyroid function (11). Attention must be given to these women since isolated fetal hyperthyroidism could develop despite maternal euthyroidism or adequately replaced hypothyroidism. On the other hand, fetal hypothyroidism could also develop if autoantibodies have a blocking activity (9). Apart from a "quantitative" change of the TRAbs, a "qualitative" change, i.e., a variation of their functional properties has been evidenced in pregnancy. Indeed switching between TBAbs and TSAbs (or *vice versa*) occurs, although unusually, in patients during L-T4 replacement therapy or ATDs treatment for Graves' disease (35). Changes from stimulating to blocking activity of TRAbs could contribute to the improvement/remission of thyrotoxicosis in pregnancy. In a study that included 15 pregnant women with Graves' disease receiving no or a low dose of ATDs and 14 healthy pregnant women, sera were tested for TRAbs, by first-generation receptor immunoassay (TBII), and for TSAbs and TBAbs by bioassays. The healthy pregnant women were all negative for TSAbs, TBII, and TBAbs. In pregnant women with Graves' disease, the TSAbs decreased significantly during pregnancy, and the TBAbs significantly increased. The TBII fluctuated and showed no correlation to the TSAb activity (54). Another study brought the same conclusion. In the sera of 13 pregnant women with Graves' disease during pregnancy and PP TBII, TSAbs and TBAbs (the last two detected by assays employing chimeric receptors) were measured. As pregnancy advanced, the TSAbs decreased and the TBAbs increased while the TBII, although fluctuating, did not change significantly. The TBAbs appeared during pregnancy

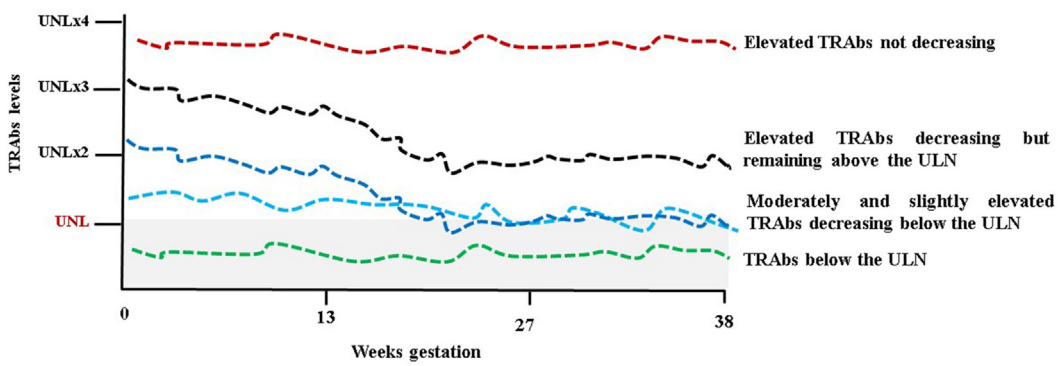


FIGURE 2 | Schematic representation of TSH receptor antibodies (TRAbs) behavior during pregnancy. UNL, upper normal limit and (x) multiples. The gray shaded area represents the normal limit.

also in women who were negative for TSAb (55). These findings were not confirmed in a study of six patients with Graves' disease receiving no or a low dose of ATDs. During pregnancy, the TBII and the TSAbs decreased gradually but increased after delivery. The TBAbs were lower than the cutoff value in early pregnancy, and further significantly decreased in four patients during pregnancy (56).

TRAbs AND FETAL–NEONATAL THYROID FUNCTION

Apart from the clinical significance of the TRAbs in determining the course of Graves' disease in pregnant women, a more relevant role is attributed to the autoantibodies in affecting the fetal and neonatal thyroid function. The fetal/neonatal thyroid dysfunction is the ideal human *in vivo* experimental system for the evaluation of the TRAbs (**Figure 3**). TRAbs easily cross the placenta from the first weeks of gestation. However, placental permeability is low early in pregnancy and increases progressively. The fetal thyroid becomes responsive to the TSH and to the TRAbs at around week 20 of gestation.

Fetal and Neonatal Hyperthyroidism

Fetal hyperthyroidism, which is the more common and expected dysfunction, develops usually at around 26 weeks, or as early as 18 weeks in severe cases (57). The prevalence of fetal hyperthyroidism is difficult to establish and several case reports have been published (58). Untreated maternal Graves' disease can lead to severe fetal hyperthyroidism and this could explain perinatal mortality of 20–45% observed before the introduction of ATDs (59, 60). Alternatively, mild fetal hyperthyroidism may not be noticed. Fetal hyperthyroidism is almost invariably followed by neonatal hyperthyroidism whose prevalence seems to be better established. Overt neonatal hyperthyroidism is reported in 1–5% of neonates born to mothers with Graves' disease (58, 61, 62). Fetal/neonatal hyperthyroidism is associated with the maternal thyroid condition (serum FT4 levels, dose of ATDs required to achieve adequate euthyroidism) and with the serum TRAbs levels (63). Several studies were aimed to establish a threshold of maternal TRAbs levels that could define the risk of fetal/neonatal hyperthyroidism. Using first-generation TRAbs assay, an increased risk of fetal/neonatal hyperthyroidism was reported if the maternal level of TBII was over 40 IU/l (normal range, <10 IU/l) or over 50% (normal range, <10–15%) in the third trimester of pregnancy (19, 64–66). In a study including 62 pregnant women with Graves' disease, TRAbs were measured by four immunoassays: first generation, second generation using porcine TSHR, second generation using human recombinant TSHR, and third generation. The first generation assay cutoff (>50%) correlated with the equivalent for second (>10 IU/L) and third (>75%) generation assay cutoff in predicting the risk of fetal–neonatal dysfunction. However, third-generation assay identified additional high-risk women whose first-generation TRAbs were below 50%. In the same study TSAbs were assayed in 20 mothers, 4 of them, having high TSAbs values ranging from 412 to 1,584%, gave birth to infants with hyperthyroidism; this was not observed in newborns born to mothers whose TSAbs were below 400%, regardless of the TBII value (46). More recently, a study of 47 neonates born to 42 mothers with measurable levels of TBII during pregnancy (assayed by second-generation immunoassay) showed that all the 9 hyperthyroid neonates were born to mothers with TRAbs values above 5 IU/L in the second trimester of pregnancy. A TBII value over 5 IU/l (which is 3.3 times the detection level of the method) during the second and third trimester of pregnancy predicted the neonatal hyperthyroidism with a sensitivity of 100% and a specificity of only 43%. Measurement of the TSAbs by bioassay showed that no mother with TSAbs below 400% gave birth to a hyperthyroid neonate (47). In summary, fetal/neonatal hyperthyroidism can be

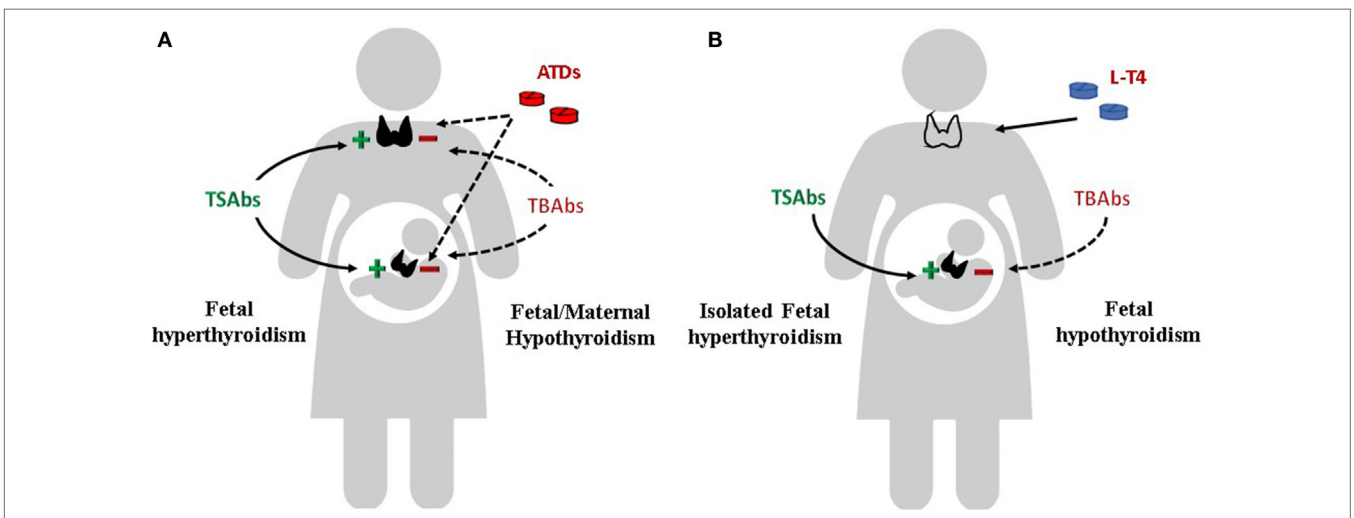


FIGURE 3 | Effects of TSH receptor antibodies and antithyroid drugs (ATDs) on maternal and fetal thyroid function. **(A)** Maternal and fetal thyroid are stimulated by TSAbs (continue line) and inhibited by ATDs and TBAbs (dotted line). If TBAbs are present, fetal as well as maternal hypothyroidism can occur. **(B)** Maternal hypothyroidism on L-T4 replacement after radioiodine therapy or thyroidectomy for Graves' disease. Isolated fetal hyperthyroidism can occur. If TBAbs are present, fetal hypothyroidism can also occur.

predicted by maternal TRAbs levels. According to guidelines, in clinical practice fetal surveillance is recommended in women with TRAbs levels exceeding three times the upper limit of normal at any time during gestation (9). Therefore, determination of TRAbs to establish the risk of fetal/neonatal hyperthyroidism is a recommendation shared by the various guidelines that have been published (8, 9, 19, 67, 68) (**Table 2**). At-risk pregnancies need to be monitored carefully, with repeated ultrasound examinations from 20 weeks of gestation onward, to screen for goiter and ultrasound findings of fetal thyroid dysfunction (58). Indeed the maternal history, the thyroid functional status, the TRAbs levels, and ultrasound parameters are the diagnostic clues for fetal hyperthyroidism. Thyroid gland enlargement is the first sign that suggests fetal hyperthyroidism and precedes fetal tachycardia (fetal heart rate >160/min). The fetal thyroid size needs to be determined by using normative data according to the gestational age (69). It is worth remembering that fetal goiter can also be observed in hypothyroid fetuses as a consequence of the maternal ATDs transfer. Peripheral thyroid vascularization, instead of diffuse increased blood flow, delayed bone maturation, and fetal heart rate <160/min, favors the diagnosis of fetal hypothyroidisms. Other ultrasound findings of hyperthyroidism are accelerated bone maturation (i.e., distal femoral center seen before 32 weeks) and intrauterine growth retardation. Fetal growth should be followed by standard gestational age sonographic parameters, in particular the abdominal circumference, since hyperthyroid fetuses are thin. The sensitivity and specificity of fetal thyroid ultrasound at 32 weeks for the diagnosis of clinically relevant fetal thyroid dysfunction are reported to be 92 and 100%, respectively, and could replace invasive and hazardous examinations, such as fetal blood collection or amniotic fluid sampling (48, 70). Early diagnosis and treatment of fetal hyperthyroidism are crucial to prevent death *in utero*, premature delivery, or congestive heart failure. Fortunately, the ATDs used to treat maternal hyperthyroidism cross the placenta, thus controlling fetal hyperthyroidism. The ATDs tend to overtreat the fetus and therefore the dose of drug given to the mother needs to be as low as possible (**Figure 3**). The monitoring of the maternal thyroid function and fetal ultrasound are the clues for the management of Graves' disease in pregnancy. A peculiar form of fetal isolated hyperthyroidism is that observed in euthyroid or hypothyroid pregnant women previously treated with surgery and/or radioiodine for GD since, as discussed above, TRAb could persist for years. Fetal/neonatal hyperthyroidism has been described in two consecutive pregnancies in a woman treated with surgery 10 years before the first pregnancy (71) and in up to 11% of women treated with radioiodine whose levels of TRAbs did not decrease during gestation regardless of the time from the radioiodine treatment to conception (72). In these patients, fetal thyroid stimulation can occur, despite maternal euthyroidism or levothyroxine replaced hypothyroidism. These are the only women who should receive block and replacement therapy in pregnancy, i.e., ATDs to treat the fetal hyperthyroidism, and levothyroxine to keep the mother's euthyroidism remembering that the placental transfer of ATDs is greater than that of levothyroxine. In a review, 11 published reports involving 13 pregnancies, the ATDs treatment of mothers whose TRAbs levels were >5-fold above normal, resulted in 13 live births while in previous pregnancies 6 serious complications (miscarriages, stillborn, or infant deaths) had been observed (73). Neonatal hyperthyroidism is suspected in newborns presenting tachycardia, hyperexcitability, and poor weight gain. Goiter, eyelid retraction and/or exophthalmos, small anterior fontanel are additional clinical clues. Congestive heart failure is one of the major immediate causes of morbidity. However, long-term complications such as craniosynostosis, microcephaly, and psychomotor disabilities may occur in severely affected newborns (58).

TABLE 2 | Indications and timing for TSH receptor antibody (TRAb) assays in pregnancy according to guidelines.

Society (reference)	Indication for TRAbs assay	Timing	TRAbs level at risk for fetal hyperthyroidism
ETA 1998 (19)	Euthyroid pregnant woman (with/without thyroid hormone substitution therapy) who has previously received radioiodine therapy or undergone thyroid surgery for Graves' disease	Early in pregnancy and in the last trimester if antibodies are present	40 U/l
Endocrine Society 2007 (67)	Current Graves' disease, history of Graves' disease and treatment with ^{131}I or thyroidectomy, previous neonate with Graves' disease	Before pregnancy or by the end of the second trimester	
Endocrine Society 2012 (8)	Current Graves' disease; history of Graves' disease and treatment with 1^{31}I or thyroidectomy before pregnancy; previous neonate with Graves' disease; previously elevated TRAb	Week 22	2- to 3-fold the normal level
ATA 2011 (68)	Past or present history of Graves' disease	Weeks 20–24	>3 times the upper limit of normal
ATA 2017 (9)	Past history of Graves' disease treated with ablation (radioiodine or surgery)	Early in pregnancy repeat determination at weeks 18–22	>3 times the upper limit of normal
	Patient on antithyroid drugs (ATDs) for treatment of Graves' hyperthyroidism when pregnancy is confirmed	Early in pregnancy	
	Patient requires treatment with ATDs for Graves' disease through mid pregnancy	Repeat determination at weeks 18–22	
	Elevated TRAb at weeks 18–22 or the mother is taking ATD in the third trimester	Repeat determination at weeks 30–34	

It is remarkable to notice that signs of hyperthyroidism may not become apparent until 2–5 days in newborns to mothers on ATDs. This is the time necessary for the ATDs to be cleared from the newborn circulation (70). Hyperthyroidism is transient and persists as long as the TRAbs become undetectable. The half-life of the TRAbs is estimated to be 2–3 weeks (74–76). Duration of treatment of infants with ATDs is most commonly 1–2 months and only exceptionally longer (47, 77). A correlation has been found between maternal TRAbs levels and neonatal hyperthyroidism. In a study of 172 pregnant women with Graves' disease neonatal hyperthyroidism developed in 6.5% of infants, most of them were born to mothers whose TRAbs levels were 30% or more (i.e., 2–5 times above the normal range) at delivery (61). In a study of 29 women with a history of Graves' disease and positive TRAbs, neonatal thyrotoxicosis developed in 17%. A TRAbs level threshold of 5UI predicted neonatal thyrotoxicosis with a sensitivity of 100%, specificity of 76.0%, positive predictive value of 40.0%, and negative predictive value of 100% (78). In a more recent study of 68 neonates born to mothers with GD, none of the infants born to TRAbs-negative mothers developed neonatal hyperthyroidism. 73% of infants born to TRAbs-positive mothers had positive TRAbs on cord blood assays, and 30% of these developed neonatal hyperthyroidism. All hyperthyroid neonates had cord blood levels of TRAbs greater than two times the upper normal level. A correlation was found between the TRAbs cord blood levels and the maternal serum TRAbs levels at term, thus confirming that the latter are a good predictor of neonatal hyperthyroidism. This was not the same for FT4 whose cord blood levels reflected fetal rather than neonatal thyroid function. The FT4 needs be revaluated on day 3–5 to establish thyrotoxicosis and the need for treatment (79).

Fetal and Neonatal Hypothyroidism

As previously mentioned, sometimes the TRAbs have a blocking effect on the TSH receptor, thus inducing fetal and neonatal hypothyroidism (**Figure 3**). Measurement of TBAbs by a bioassay in dried neonatal blood specimens obtained from 788 neonates identified as congenital hypothyroidism at the neonatal screening program in US demonstrated potent TSHR-blocking activity in 11 cases. The 11 babies were born to 9 mothers, all of whom were receiving thyroid replacement because of autoimmune hypothyroidism, and 3 had been treated initially for Graves' disease. TPO antibodies, although detectable in all mothers, did not predict the neonatal thyroid dysfunction, while the presence of TBAbs was confirmed in the serum of eight mothers: all newborns had transient congenital hypothyroidism. The author estimated the prevalence of TBAbs-induced congenital hypothyroidism in the order of 1 in 180,000, or about 2% of all cases (80). In a large series of newborns screened for congenital hypothyroidism in Wales (375 cases identified over 966,969 infants screened), 6 (1.6%) were found to have transient congenital hypothyroidism due to maternal TBAbs. All the mothers were hypothyroid on levothyroxine replacement therapy or were diagnosed with hypothyroidism after the reported elevation of TSH in their infants (81). The presence of TBAbs has been advocated to explain the delayed onset of neonatal hyperthyroidism in newborns to mother with Graves' disease harboring both stimulating and blocking antibodies (82). In this situation, it can be hypothesized that differences in the receptor affinity, as well as in the clearance rate of the two populations of antibodies, determine the clinical course of thyroid dysfunction in the neonate (35).

TRAbs IN THE POSTPARTUM

During the PP a rebound reaction to the pregnancy-associated immunosuppression is observed and this explains the aggravation of autoimmune diseases during the puerperium. The levels of TRAbs could increase and women who experienced remission during late pregnancy, as well as women who were in remission after the ATDs course before pregnancy, could experience relapse in the PP. After ATDs withdrawal, relapse of Graves' hyperthyroidism was observed in 84% of women who had further pregnancies compared to 56% of women who did not remain pregnant. The number of pregnancies after ATDs cessation was significantly correlated with the risk of relapse. The relapse of Graves' hyperthyroidism occurred between 4 and 8 months after delivery (83). On the other hand, *de novo* onset of Graves' disease after pregnancy has been reported in 7–8% (84). Few studies have focused on establishing a predictive role of the TRAbs positivity early in pregnancy for postpartum onset of Graves' thyrotoxicosis. In 71 women with positive antithyroid microsomal antibody (MCAb), 10% showed positive TRAbs (both TBII and TSAb) in early pregnancy, although without any thyroid dysfunction; 71% of them developed Graves' disease PP, none of the TSAbs-negative subjects developed Graves' thyrotoxicosis. Various types of thyroid dysfunction as a result of postpartum autoimmune thyroiditis were found in 62% of MCAb-positive women (85). In a further study of 38 pregnant women who were positive for TPOAb, 10% were positive for TSAbs measured by a sensitive bioassay. PP Graves' hyperthyroidism developed in 50% of TSAbs-positive women. These findings indicate that the third-generation TRAbs assay was not useful; however, a sensitive TSAbs bioassay was moderately useful for predicting the PP onset of Graves' hyperthyroidism (86). Apart from the possible role played by the TRAbs assay in early pregnancy in predicting the risk of developing the disease in the puerperium, antibody testing plays an important role in clinical practice to differentiate Graves' hyperthyroidism from thyrotoxic phase of postpartum thyroid dysfunction (PPTD). The PTDD occurs in approximately 5–10% of women in the general population within 1 year of delivery and that is significantly higher than the prevalence of Graves' disease in childbearing age. A differential diagnosis is essential given the two conditions differ significantly in the course as well as in the treatment. In a series of 42 women developing PP thyrotoxicosis, 86% had PPDT and 24% had Graves' disease. TRAbs measured with third-generation receptor assay were positive in all patients with Graves' disease and negative in all patients with the PTTD; the latter also showing low thyroid blood flow measured quantitatively by color flow Doppler ultrasonography. The PPTD occurred earlier (3 months or less earlier after delivery), while Graves' disease developed at 6 months or later (87). In another study, the second-generation assay for TRAbs was useful to differentiate the relapse of Graves' thyrotoxicosis from development of painless thyroiditis in patients who seemed to be in remission

after ATDs treatment for Graves' disease. 85.7% of 14 patients with a relapse of Graves' thyrotoxicosis were positive for TRAbs, and 91.7% of 12 patients who developed painless thyroiditis after ATDs treatment for Graves' disease were negative for TRAbs (88). The clinical relevance of these observations is that in women with history of Graves' disease, thyroid function monitoring and TRAbs measurement are needed in the PP, irrespective of the course the disease takes during pregnancy. Beyond the role of the changes in the autoimmune response occurring during gestation and in the PP, it has to be highlight that pregnancy and delivery have to be considered stressful events, which could have, on their own, a causative role in the onset, relapse or exacerbation of Graves' disease. In a paradigmatic case report, a combination of stressful life events and pregnancy is reported. In a young woman, the onset of Graves' disease shortly followed an emotional stress. The woman was treated with ATDs and experienced exacerbation of hyperthyroidism during her first pregnancy and 9 months after her first delivery. In both occasions, a stressful life event was retraced in her history (89). It has been reported that there exist patients with Graves' disease in whom onset, exacerbation or relapse of hyperthyroidism are systematically preceded by at least one stressful event (90). Very recently, in these group of patients, HLA typing has demonstrated that both HLA class I and class II molecules are associated with stress-triggered Graves' with certain HLA alleles and loci predisposing, while others protecting from stress-related Graves' disease (91).

CONCLUSION

Thyroid diseases in pregnancy affect the physiological mechanisms that allow the thyroid function to be adequate both for maternal and fetal requirements. Thyroid autoimmune diseases are the most common cause of thyroid dysfunction in childbearing women and thyroid autoantibodies are associated with several adverse maternal and fetal outcomes. TRAbs, which are the pathogenetic hallmark of Graves' disease, present peculiar challenges in pregnancy. In fact, unlike Tg and TPO autoantibodies, they can directly affect contemporarily and/or independently, fetal as well as maternal thyroid function. On the other hand, pregnancy-related immunosuppression in most cases reduces the maternal levels of antibodies. Information about the TRAbs status (presence, levels), on their behavior (changes after radioiodine therapy or surgery and during gestation and PP) and on their multifaceted properties (stimulating or blocking activity) are essential for the preconceptional counseling as well as for the therapy of Graves' disease during gestation and in the PP. This information is also crucial for the prediction and for the management of fetal thyroid dysfunction. The preservation of maternal and fetal euthyroidism is the challenge of the management of Graves' disease in pregnancy.

AUTHOR CONTRIBUTIONS

IB: substantial contributions to the conception and design of the work; reviewing the literature; drafting the work; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. CG: substantial contributions to the design of the work; revising the work critically for important intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. GN: substantial contributions to the conception of the work; revising it critically for intellectual content; final approval of the version to be published; and agreement to be accountable for all aspects of the work.

REFERENCES

1. Glinoer D. Regulation of thyroid function in pregnancy: pathways of endocrine adaptation from physiology to pathology. *Endocr Rev* (1997) 18:404–33. doi:10.1210/edrv.18.3.0300
2. Vermiglio F, Moleti M, Trimarchi F. Thyroid diseases in pregnancy. In: Monaco F, editor. *Thyroid Diseases*. Boca Raton, FL: CRC Press (2012). p. 425–40.
3. Glinoer D. The importance of iodine nutrition during pregnancy. *Public Health Nutr* (2007) 10:1542–6. doi:10.1017/S1368980007360886
4. Lazarus JH. Thyroid function in pregnancy. *Br Med Bull* (2011) 97:137–48. doi:10.1093/bmb/ldq039
5. Roti E, Gardini E, Minelli R, Bianconi L, Flisi M. Thyroid function evaluation by different commercially available free thyroid hormone measurement kits in term pregnant women and their newborns. *J Endocrinol Invest* (1991) 14:1–9. doi:10.1007/BF03350244
6. de Escobar GM, Obregón MJ, del Rey FE. Maternal thyroid hormones early in pregnancy and fetal brain development. *Best Pract Res Clin Endocrinol Metab* (2004) 18:225–48. doi:10.1016/j.beem.2004.03.012
7. Pearce EN. Thyroid disorders during pregnancy and postpartum. *Best Pract Res Clin Obstet Gynaecol* (2015) 29:700–6. doi:10.1016/j.bpobgyn.2015.04.007
8. De Groot L, Abalovich M, Alexander EK, Amino N, Barbour L, Cobin RH, et al. Management of thyroid dysfunction during pregnancy and postpartum: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* (2012) 97:2543–65. doi:10.1210/jc.2011-2803
9. Alexander EK, Pearce EN, Brent GA, Brown RS, Chen H, Dosiou C, et al. 2017 guidelines of the American Thyroid Association for the diagnosis and management of thyroid disease during pregnancy and the postpartum. *Thyroid* (2017) 27:315–89. doi:10.1089/thy.2016.0457
10. Stagnaro-Green A, Roman SH, Cobin RH, el-Harazy E, Alvarez-Marfany M, Davies TF. Detection of at-risk pregnancy by means of highly sensitive assays for thyroid autoantibodies. *JAMA* (1990) 264:1422–5. doi:10.1001/jama.1990.03450110068029
11. Balucan FS, Morshed SA, Davies TF. Thyroid autoantibodies in pregnancy: their role, regulation and clinical relevance. *J Thyroid Res* (2013) 2013:182472. doi:10.1155/2013/182472
12. Negro R, Mestman JH. Thyroid disease in pregnancy. *Best Pract Res Clin Endocrinol Metab* (2011) 25:927–43. doi:10.1016/j.beem.2011.07.010
13. Cooper DS, Laurberg P. Hyperthyroidism in pregnancy. *Lancet Diabetes Endocrinol* (2013) 1:238–49. doi:10.1016/S2213-8587(13)70086-X
14. Orito Y, Oku H, Kubota S, Amino N, Shimogaki K, Hata M, et al. Thyroid function in early pregnancy in Japanese healthy women: relation to urinary iodine excretion, emesis, and fetal and child development. *J Clin Endocrinol Metab* (2009) 94:1683–8. doi:10.1210/jc.2008-2111
15. Yeo CP, Khoo DH, Eng PH, Tan HK, Yo SL, Jacob W. Prevalence of gestational thyrotoxicosis in Asian women evaluated in the 8th to 14th weeks of pregnancy: correlations with total and free beta human chorionic gonadotrophin. *Clin Endocrinol (Oxf)* (2001) 55:391–8. doi:10.1046/j.1365-2265.2001.01353.x
16. Andersen SL, Olsen J, Carlé A, Laurberg P. Hyperthyroidism incidence fluctuates widely in and around pregnancy and is at variance with some other

autoimmune diseases: a Danish population-based study. *J Clin Endocrinol Metab* (2015) 100:1164–71. doi:10.1210/jc.2014-3588
17. Laurberg P, Andersen SL. Pregnancy and the incidence, diagnosing and therapy of Graves' disease. *Eur J Endocrinol* (2016) 175:R219–30. doi:10.1530/EJE-16-0410
18. Amino N, Tanizawa O, Mori H, Iwatani Y, Yamada T, Kurachi K, et al. Aggravation of thyrotoxicosis in early pregnancy and after delivery in Graves' disease. *J Clin Endocrinol Metab* (1982) 55:108–12. doi:10.1210/jcem-55-1-108
19. Laurberg P, Nygaard B, Glinoer D, Grussendorf M, Orgiazzi J. Guidelines for TSH-receptor antibody measurements in pregnancy: results of an evidence-based symposium organized by the European Thyroid Association. *Eur J Endocrinol* (1998) 139:584–96. doi:10.1530/eje.0.1390584
20. Easterling TR, Schmucker BC, Carlson KL, Millard SP, Benedetti TJ. Maternal hemodynamics in pregnancies complicated by hyperthyroidism. *Obstet Gynecol* (1991) 78:348–52.
21. Millar LK, Wing DA, Leung AS, Koonings PP, Montoro MN, Mestman JH. Low birth weight and preeclampsia in pregnancies complicated by hyperthyroidism. *Obstet Gynecol* (1994) 84:946–9.
22. Phoojaroenchanachai M, Sriussadaporn S, Peerapatdit T, Vannasaeng S, Nitiyanant W, Boonnamsiri V, et al. Effect of maternal hyperthyroidism during late pregnancy on the risk of neonatal low birth weight. *Clin Endocrinol* (2001) 54:365–70. doi:10.1046/j.1365-2265.2001.01224.x
23. Aggarawal N, Suri V, Singla R, Chopra S, Sikka P, Shah VN, et al. Pregnancy outcome in hyperthyroidism: a case control study. *Gynecol Obstet Invest* (2014) 77:94–9. doi:10.1159/000357615
24. Clementi M, Di Gianantonio E, Pelo E, Mammi I, Basile RT, Tenconi R. Methimazole embryopathy: delineation of the phenotype. *Am J Med Genet* (1999) 83:43–6. doi:10.1002/(SICI)1096-8628(19990305)83:1<43::AID-AJMG8>3.0.CO;2-C
25. Di Gianantonio E, Schaefer C, Mastroiacovo PP, Cournot MP, Benedicenti F, Reuvers M, et al. Adverse effects of prenatal methimazole exposure. *Teratology* (2001) 64:262–6. doi:10.1002/tera.1072
26. Yoshihara A, Noh J, Yamaguchi T, Ohye H, Sato S, Sekiya K, et al. Treatment of Graves' disease with antithyroid drugs in the first trimester of pregnancy and the prevalence of congenital malformation. *J Clin Endocrinol Metab* (2012) 97:2396–403. doi:10.1210/jc.2011-2860
27. Andersen SL, Olsen J, Wu CS, Laurberg P. Birth defects after early pregnancy use of antithyroid drugs: a Danish nationwide study. *J Clin Endocrinol Metab* (2013) 98:4373–81. doi:10.1210/jc.2013-2831
28. Laurberg P, Andersen SL. Antithyroid drug use in pregnancy and birth defects: why some studies find clear associations, and some studies report none. *Thyroid* (2015) 25:1185–90. doi:10.1089/thy.2015.0182
29. Mortimer RH, Cannell GR, Addison RS, Johnson LP, Roberts MS, Bernus I. Methimazole and propylthiouracil equally cross the perfused human term placental lobule. *J Clin Endocrinol Metab* (1997) 82:3099–102. doi:10.1210/jcem.82.9.4210
30. Ando T, Latif R, Davies TF. Thyrotropin receptor antibodies: new insights into their actions and clinical relevance. *Best Pract Res Clin Endocrinol Metab* (2005) 19:33–52. doi:10.1016/j.beem.2004.11.005
31. Michalek K, Morshed SA, Latif R, Davies TF. TSH receptor autoantibodies. *Autoimmun Rev* (2009) 9:113–6. doi:10.1016/j.autrev.2009.03.012
32. Kahaly GJ, Diana T. TSH receptor antibody functionality and nomenclature. *Front Endocrinol* (2017) 8:28. doi:10.3389/fendo.2017.00028
33. Adams DD. The presence of an abnormal thyroid-stimulating hormone in the serum of some thyrotoxic patients. *J Clin Endocrinol Metab* (1958) 18:699–712. doi:10.1210/jcem-18-7-699
34. Giuliani C, Monaco F. Adult primary hypothyroidism. In: Monaco F, editor. *Thyroid Diseases*. Boca Raton, FL: CRC Press (2012). p. 171–94.
35. McLachlan SM, Rapoport B. Thyrotropin-blocking autoantibodies and thyroid-stimulating autoantibodies: potential mechanisms involved in the pendulum swinging from hypothyroidism to hyperthyroidism or vice versa. *Thyroid* (2013) 23:14–24. doi:10.1089/thy.2012.0374
36. Barbesino G, Tomer Y. Clinical utility of TSH receptor antibodies. *J Clin Endocrinol Metab* (2013) 98:2247–55. doi:10.1210/jc.2012-4309
37. Lytton SD, Kahaly GJ. Bioassays for TSH-receptor autoantibodies: an update. *Autoimmun Rev* (2010) 10:116–22. doi:10.1016/j.autrev.2010.08.018
38. Tozzoli R, Bagnasco M, Giavarina D, Bizzarro N. TSH receptor autoantibody immunoassay in patients with Graves' disease: improvement of diagnostic accuracy over different generations of methods, systematic review and meta-analysis. *Autoimmun Rev* (2012) 12:107–13. doi:10.1016/j.autrev.2012.07.003
39. Giuliani C, Saji M, Bucci I, Napolitano G. Bioassays for TSH receptor autoantibodies, from FRTL-5 cells to TSH receptor-LH/CG receptor chimeras: the contribution of Leonard D. Kohn. *Front Endocrinol* (2016) 7:103. doi:10.3389/fendo.2016.00103
40. Giuliani C, Cerrone D, Harii N, Thornton M, Kohn LD, Dagia NM, et al. A TSHr-LH/CGr chimera that measures functional TSAb in Graves' disease. *J Clin Endocrinol Metab* (2012) 97:E1106–15. doi:10.1210/jc.2011-2893
41. Giuliani C, Cerrone D, Harii N, Thornton M, Kohn LD, Dagia NM, et al. A TSHr-LH/CGr chimera that measures functional thyroid-stimulating autoantibodies (TSAb) can predict remission or recurrence in Graves' patients undergoing antithyroid drug (ATD) treatment. *J Clin Endocrinol Metab* (2012) 97:E1080–7. doi:10.1210/jc.2011-2897
42. Li Y, Kim J, Diana T, Klasen R, Olivo PD, Kahaly GJ. A novel bioassay for anti-thyrotrophin receptor autoantibodies detects both thyroid-blocking and stimulating activity. *Clin Exp Immunol* (2013) 173:390–7. doi:10.1111/cei.12129
43. Chan GW, Mandel SJ. Therapy insight: management of Graves' disease during pregnancy. *Nat Clin Pract Endocrinol Metab* (2007) 3:470–8. doi:10.1038/ncpendmet0508
44. McKenzie JM, Zakarija M. Fetal and neonatal hyperthyroidism and hypothyroidism due to maternal TSH receptor antibodies. *Thyroid* (1992) 2:155–9. doi:10.1089/thy.1992.2.155
45. Gonzalez-Jimenez A, Fernandez-Soto ML, Escobar-Jimenez F, Glinoer D, Navarrete L. Thyroid function parameters and TSH-receptor antibodies in healthy subjects and Graves' disease patients: a sequential study before, during and after pregnancy. *Thyroidology* (1993) 5:13–20.
46. Kamijo K. TSH-receptor antibodies determined by the first, second and third generation assays and thyroid-stimulating antibody in pregnant patients with Graves' disease. *Endocr J* (2007) 54:619–24. doi:10.1507/endocrj.K06-196
47. Abeillon-du Payrat J, Chikh K, Bossard N, Bretones P, Gaucherand P, Claris O, et al. Predictive value of maternal second-generation thyroid-binding inhibitory immunoglobulin assay for neonatal autoimmune hyperthyroidism. *Eur J Endocrinol* (2014) 171:451–60. doi:10.1530/EJE-14-0254
48. Luton D, Le Gac I, Vuillard E, Castanet M, Guibourdenche J, Noel M, et al. Management of Graves' disease during pregnancy: the key role of fetal thyroid gland monitoring. *J Clin Endocrinol Metab* (2005) 90:6093–8. doi:10.1210/jc.2004-2555
49. Atkinson S, McGregor AM, Kendall-Taylor P, Peterson MM, Smith BR. Effect of radioiodine on stimulatory activity of Graves' immunoglobulins. *Clin Endocrinol (Oxf)* (1982) 16:537–43. doi:10.1111/j.1365-2265.1982.tb03170.x
50. Chiappori A, Villalta D, Bossert I, Ceresola EM, Lanaro D, Schiavo M, et al. Thyrotropin receptor autoantibody measurement following radiometabolic treatment of hyperthyroidism: comparison between different methods. *J Endocrinol Invest* (2010) 33:197–201. doi:10.1007/BF03346581
51. Laurberg P, Wallin G, Tallstedt L, AbrahamNordling M, Lundell G, Torring O. TSH-receptor autoimmunity in Graves' disease after therapy with antithyroid drugs, surgery, or radioiodine: a 5-year prospective randomized study. *Eur J Endocrinol* (2008) 158:69–75. doi:10.1530/EJE-07-0450
52. Yoshioka W, Miyauchi A, Ito M, Kudo T, Tamai H, Nishihara E, et al. Kinetic analyses of changes in serum TSH receptor antibody values after total thyroidectomy in patients with Graves' disease. *Endocr J* (2016) 63:179–85. doi:10.1507/endocrj.EJ15-0492
53. Laurberg P, Bournaud C, Karmisholt J, Orgiazzi J. Management of Graves' hyperthyroidism in pregnancy: focus on both maternal and foetal thyroid function, and caution against surgical thyroidectomy in pregnancy. *Eur J Endocrinol* (2009) 160:1–8. doi:10.1530/EJE-08-0663
54. Kung AW, Jones BM. A change from stimulatory to blocking antibody activity in Graves' disease during pregnancy. *J Clin Endocrinol Metab* (1998) 83:514–8. doi:10.1210/jc.83.2.514
55. Kung AW, Lau KS, Kohn LD. Epitope mapping of TSH receptor-blocking antibodies in Graves' disease that appear during pregnancy. *J Clin Endocrinol Metab* (2001) 86:3647–53. doi:10.1210/jc.86.8.3647
56. Amino N, Izumi Y, Hidaka Y, Takeoka K, Nakata Y, Tatsumi KI, et al. No increase of blocking type anti-thyrotropin receptor antibodies during pregnancy in patients with Graves' disease. *J Clin Endocrinol Metab* (2003) 88:5871–4. doi:10.1210/jc.2003-030971

57. Donnelly MA, Wood C, Casey B, Hobbins J, Barbour LA. Early severe fetal Graves' disease in a mother after thyroid ablation and thyroidectomy. *Obstet Gynecol* (2015) 125:1059–62. doi:10.1097/AOG.0000000000000582
58. Polak M, Le Gac I, Vuillard E, Guibourdenche J, Leger J, Toubert ME, et al. Fetal and neonatal thyroid function in relation to maternal Graves' disease. *Best Pract Res Clin Endocrinol Metab* (2004) 18:289–302. doi:10.1016/j.beem.2004.03.009
59. Gardner-Hill H. Pregnancy complicating simple goiter and Graves' disease. *Lancet* (1929) 213:120–4. doi:10.1016/S0140-6736(00)82433-2
60. Chopra IJ. Fetal and neonatal hyperthyroidism. *Thyroid* (1992) 2:161–3. doi:10.1089/thy.1992.2.161
61. Mitsuda N, Tamaki H, Amino N, Hosono T, Miyai K, Tanizawa O. Risk factors for developmental disorders in infants born to women with Graves disease. *Obstet Gynecol* (1992) 80:359–64.
62. Zakaraija M, McKenzie JM, Hoffman WH. Prediction and therapy of intrauterine and late-onset neonatal hyperthyroidism. *J Clin Endocrinol Metab* (1986) 62:368–71. doi:10.1210/jcem-62-2-368
63. Uenaka M, Tanimura K, Tairaku S, Morioka I, Ebina Y, Yamada H. Risk factors for neonatal thyroid dysfunction in pregnancies complicated by Graves' disease. *Eur J Obstet Gynecol Reprod Biol* (2014) 177:89–93. doi:10.1016/j.ejogrb.2014.03.007
64. Clavel S, Madec AM, Bornet H, Deviller P, Stefanutti A, Orgiazzi J. Anti TSH-receptor antibodies in pregnant patients with autoimmune thyroid disorder. *Br J Obstet Gynaecol* (1990) 97:1003–8. doi:10.1111/j.1471-0528.1990.tb02472.x
65. Wallace C, Couch R, Ginsberg J. Fetal thyrotoxicosis: a case report and recommendations for prediction, diagnosis, and treatment. *Thyroid* (1995) 5:125–8. doi:10.1089/thy.1995.5.125
66. Matsuura N, Konishi J, Fujieda K, Kasagi K, Iida Y, Hirasawa M, et al. TSH-receptor antibodies in mothers with Graves' disease and outcome in their offspring. *Lancet* (1988) 1:14–7. doi:10.1016/S0140-6736(88)91001-X
67. Abalovich M, Amino N, Barbour LA, Cobin RH, De Groot LJ, Glinoer D, et al. Management of thyroid dysfunction during pregnancy and postpartum: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab* (2007) 92:S1–47. doi:10.1210/jc.2007-0141
68. Stagnaro-Green A, Abalovich M, Alexander E, Azizi F, Mestman J, Negro R, et al. Guidelines of the American Thyroid Association for the diagnosis and management of thyroid disease during pregnancy and postpartum. *Thyroid* (2011) 21:1081–125. doi:10.1089/thy.2011.0087
69. Ranzini AC, Ananth CV, Smulian JC, Kung M, Limbachia A, Vintzileos AM. Ultrasonography of the fetal thyroid: normograms based on biparietal diameter and gestational age. *J Ultrasound Med* (2001) 20:613–7. doi:10.7863/jum.2001.20.6.613
70. Léger J. Management of fetal and neonatal Graves' disease. *Horm Res Paediatr* (2017) 87:1–6. doi:10.1159/000453065
71. Dierickx I, Decallonne B, Billen J, Vanhole C, Lewi L, De Catte L, et al. Severe fetal and neonatal hyperthyroidism years after surgical treatment of maternal Graves' disease. *J Obstet Gynaecol* (2014) 34:117–22. doi:10.3109/01443615.2013.831044
72. Hamada N, Momotani N, Ishikawa N, Yoshimura Noh J, Okamoto Y, Konishi T, et al. Persistent high TRAb values during pregnancy predict increased risk of neonatal hyperthyroidism following radioiodine therapy for refractory hyperthyroidism. *Endocr J* (2011) 58:55–8. doi:10.1507/endocrj.K10E-123
73. McNab T, Ginsberg J. Use of anti-thyroid drugs in euthyroid pregnant women with previous Graves' disease. *Clin Invest Med* (2005) 28:127–31.
74. McKenzie JM. Neonatal Graves' disease. *J Clin Endocrinol Metab* (1964) 24:660–8. doi:10.1210/jcem-24-7-660
75. Skuza KA, Sills IN, Stene M, Rapaport R. Prediction of neonatal hyperthyroidism in infants born to mothers with Graves disease. *J Pediatr* (1996) 128:264–8. doi:10.1016/S0022-3476(96)70405-5
76. Karpman BA, Rapoport B, Filetti S, Fisher DA. Treatment of neonatal hyperthyroidism due to Graves' disease with sodium ipodate. *J Clin Endocrinol Metab* (1987) 64:119–23. doi:10.1210/jcem-64-1-119
77. van der Kaay DC, Wasserman JD, Palmert MR. Management of neonates born to mothers with Graves' disease. *Pediatrics* (2016) 137(4). doi:10.1542/peds.2015-1878
78. Peleg D, Cada S, Peleg A, Ben-Ami M. The relationship between maternal serum thyroid-stimulating immunoglobulin and fetal and neonatal thyrotoxicosis. *Obstet Gynecol* (2002) 99:1040–3. doi:10.1016/S0029-7844(02)01961-0
79. Besançon A, Beltrand J, Le Gac I, Luton D, Polak M. Management of neonates born to women with Graves' disease: a cohort study. *Eur J Endocrinol* (2014) 170:855–62. doi:10.1530/EJE-13-0994
80. Brown RS, Bellisario RL, Botero D, Fournier L, Abrams CA, Cowger ML, et al. Incidence of transient congenital hypothyroidism due to maternal thyrotropin receptor-blocking antibodies in over one million babies. *J Clin Endocrinol Metab* (1996) 81:1147–51. doi:10.1210/jcem.81.3.8772590
81. Evans C, Gregory JW, Barton J, Bidder C, Gibbs J, Pryce R, et al. Transient congenital hypothyroidism due to thyroid-stimulating hormone receptor blocking antibodies: a case series. *Ann Clin Biochem* (2011) 48:386–90. doi:10.1258/acb.2011.011007
82. Zakarija M, McKenzie JM, Munro DS. Immunoglobulin G inhibitor of thyroid-stimulating antibody is a cause of delay in the onset of neonatal Graves' disease. *J Clin Invest* (1983) 72:1352–6. doi:10.1172/JCI111091
83. Rotondi M, Cappelli C, Pirali B, Pirola I, Magri F, Fonte R, et al. The effect of pregnancy on subsequent relapse from Graves' disease after a successful course of antithyroid drug therapy. *J Clin Endocrinol Metab* (2008) 93:3985–8. doi:10.1210/jc.2008-0966
84. Rotondi M, Pirali B, Lodigiani S, Bray S, Leporati P, Chytiris S, et al. The post partum period and the onset of Graves' disease: an overestimated risk factor. *Eur J Endocrinol* (2008) 159:161–5. doi:10.1530/EJE-08-0236
85. Hidaka Y, Tamaki H, Iwatani Y, Tada H, Mitsuda N, Amino N. Prediction of post-partum Graves' thyrotoxicosis by measurement of thyroid stimulating antibody in early pregnancy. *Clin Endocrinol (Oxf)* (1994) 41:15–20. doi:10.1111/j.1365-2265.1994.tb03778.x
86. Ide A, Amino N, Nishihara E, Kudo T, Ito M, Kimura Y, et al. Partial prediction of postpartum Graves' thyrotoxicosis by sensitive bioassay for thyroid-stimulating antibody measured in early pregnancy. *Endocr J* (2016) 63:929–32. doi:10.1507/endocrj.EJ16-0296
87. Ide A, Amino N, Kang S, Yoshioka W, Kudo T, Nishihara E, et al. Differentiation of postpartum Graves' thyrotoxicosis from postpartum destructive thyrotoxicosis using antithyrotropin receptor antibodies and thyroid blood flow. *Thyroid* (2014) 24:1027–31. doi:10.1089/thy.2013.0585
88. Izumi Y, Takeoka K, Amino N. Usefulness of the 2nd generation assay for anti-TSH receptor antibodies to differentiate relapse of Graves' thyrotoxicosis from development of painless thyroiditis after antithyroid drug treatment for Graves' disease. *Endocr J* (2005) 52:493–7. doi:10.1507/endocrj.52.493
89. Vita R, Lapa D, Vita G, Trimarchi F, Benvenga S. A patient with stress-related onset and exacerbations of Graves disease. *Nat Clin Pract Endocrinol Metab* (2009) 5:55–61. doi:10.1038/ncpendmet1006
90. Vita R, Lapa D, Trimarchi F, Benvenga S. Stress triggers the onset and the recurrences of hyperthyroidism in patients with Graves' disease. *Endocrine* (2015) 48:254–63. doi:10.1007/s12020-014-0289-8
91. Vita R, Lapa D, Trimarchi F, Vita G, Fallahi P, Antonelli A, et al. Certain HLA alleles are associated with stress-triggered Graves' disease and influence its course. *Endocrine* (2017) 55:93–100. doi:10.1007/s12020-016-0909-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 23 August 2016
doi: 10.3389/fendo.2016.00120

Thyrotropin Receptor Epitope and Human Leukocyte Antigen in Graves' Disease

Hidefumi Inaba[1], Leslie J. De Groot[2] and Takashi Akamizu[1]*

[1] The First Department of Medicine, Wakayama Medical University, Wakayama, Japan, [2] Department of Cellular and Molecular Biology, University of Rhode Island, Providence, RI, USA

Graves' disease (GD) is an organ-specific autoimmune disease, and thyrotropin (TSH) receptor (TSHR) is a major autoantigen in this condition. Since the extracellular domain of human TSHR (TSHR-ECD) is shed into the circulation, TSHR-ECD is a preferentially immunogenic portion of TSHR. Both genetic factors and environmental factors contribute to development of GD. Inheritance of human leukocyte antigen (HLA) genes, especially HLA-DR3, is associated with GD. TSHR-ECD protein is endocytosed into antigen-presenting cells (APCs), and processed to TSHR-ECD peptides. These peptide epitopes bind to HLA-class II molecules, and subsequently the complex of HLA-class II and TSHR-ECD epitope is presented to CD4+ T cells. The activated CD4+ T cells secrete cytokines/chemokines that stimulate B-cells to produce TSAb, and in turn hyperthyroidism occurs. Numerous studies have been done to identify T- and B-cell epitopes in TSHR-ECD, including (1) *in silico*, (2) *in vitro*, (3) *in vivo*, and (4) clinical experiments. Murine models of GD and HLA-transgenic mice have played a pivotal role in elucidating the immunological mechanisms. To date, linear or conformational epitopes of TSHR-ECD, as well as the molecular structure of the epitope-binding groove in HLA-DR, were reported to be related to the pathogenesis in GD. Dysfunction of central tolerance in the thymus, or in peripheral tolerance, such as regulatory T cells, could allow development of GD. Novel treatments using TSHR antagonists or mutated TSHR peptides have been reported to be effective. We review and update the role of immunogenic TSHR epitopes and HLA in GD, and offer perspectives on TSHR epitope specific treatments.

Keywords: TSH receptor, HLA, Graves' disease, epitope, anti-TSHR-antibody

OPEN ACCESS

Edited by:
Bernadette Biondi,
University of Naples Federico II, Italy

Reviewed by:
Angela Lombardi,
Icahn School of Medicine at Mount Sinai, USA
Roberto Vita,
University of Messina, Italy

****Correspondence:***
Hidefumi Inaba
inaba@wakayama-med.ac.jp

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *06 July 2016*
Accepted: *12 August 2016*
Published: *23 August 2016*

Citation:
Inaba H, De Groot LJ and Akamizu T (2016) Thyrotropin Receptor Epitope and Human Leukocyte Antigen in Graves' Disease. Front. Endocrinol. 7:120. doi: 10.3389/fendo.2016.00120

INTRODUCTION

Autoimmune thyroid diseases (AITDs) are organ-specific autoimmune diseases with multiple etiologies (1) (**Figure 1**). Graves' disease (GD) and Hashimoto's thyroiditis (HT) are two major components of AITDs. When individuals having susceptible genetic background are exposed to environmental factors (e.g., iodine, smoking, infections, and stress, and others so far undisclosed), thyroid autoantigens break "self-tolerance" and AITDs develop (2). Thyroid autoantigens, such as thyroglobulin (Tg), thyrotropin (TSH) receptor (TSHR), thyroid peroxidase (TPO), and NIS have increased immunogenicity when they are iodinated, and glycosylated. Tg and TSHR have genetic polymorphisms that may predispose to GD (1). Specific polymorphisms of other genes [e.g., human leukocyte antigen (HLA), cytotoxic T-lymphocytes antigen (CTLA-4), CD40] are clearly associated

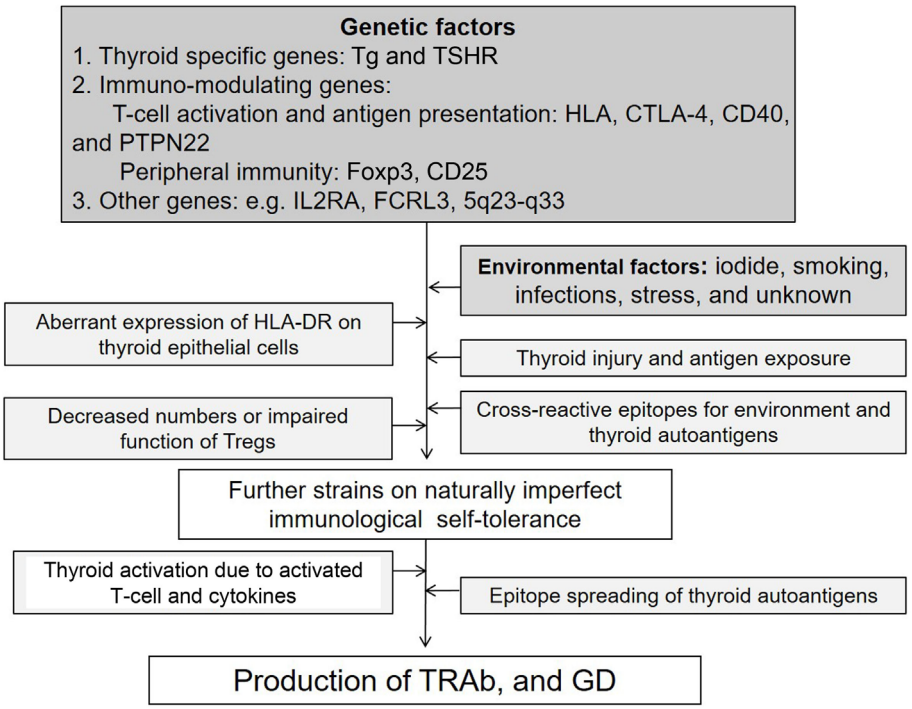


FIGURE 1 | **Factors possibly contributing to the etiology of Graves' disease (GD)**.

with GD (3–6). GD is characterized by hyperthyroidism caused by stimulatory anti-TSHR antibodies (TRAb, TSAb, TSI) (7). TSHR peptide epitopes bound to HLA-class II are presented by antigen-presenting cells (APCs) to CD4+ T cells (**Figure 2**). Interaction by the complex of TSHR epitope, HLA-class II molecule, and T-cell receptor (TCR) is modified through binding of CD40 ligand to CD40 and of CTLA-4 to B7 (3–6). TSHR epitopes bound to HLA-class II presented on the surface of APC are the most crucial factor to determine immunogenicity. Various approaches to identify the TSHR epitopes have involved *in silico*, *in vitro*, *in vivo*, and clinical studies, and some TSHR epitope clusters were reported (7, 8). Thyroid function is regulated by not only TRAb but also cell-mediated immunity (9). Two major regulations (central and peripheral) maintain self-tolerance (2). We review the immunogenic mechanisms of GD in association with TSHR and HLA, and discuss future therapeutic approaches.

TSHR AND GD

TSH receptor is one of a family of glycoprotein-coupled hormone receptors, and was cloned in 1990 (10). TSHR is indispensable for TSH signal transduction, production of thyroid hormone and Tg, and proliferation of thyroid follicular cells. TSHR consists of an extracellular domain (ECD: amino acids 1–418), a seven transmembrane domain (7TMD: 418–683) and an intracellular domain (11). ECD is also divided into Leucine-rich repeat domain (LRR: 1–276) and a hinge region (277–418). The region around 7TMD is referred to as serpentine domain (11). Upon TSHR activation, TSH or TRAb binds to TSHR, and signal is transduced through 7TMD into Gαs. Recently, Brüser et al. found that a peptide named P10 (TSHR-405-FNPCEDIMGY-414) located in C-terminus of TSHR-ECD, is conserved in different GPHRs-ECD and different species. They found that P10 can activate TSHR *in vitro*, and suggested that P10 isomerizes and induces structural changes in the 7TMD, triggering Gαs activation (12) on TSHR-ECD ligand binding. Schaarschmidt et al. proposed that the re-arrangement of the ECD (extracellular loop 1) was critical for TSHR activation (13). TSHR is alternatively divided into an A-subunit (amino acids 1–302), C-domain (303–367), and B-subunit (368–764) (14). Deletion of 50 amino acids in C-domain (residues 317–366) had no effect on TSH binding or on TSH and TSAb-stimulating activities (15). After C-domain is physiologically cleaved (15), A-subunit (residues 22–289) is shed into the circulation (14). Thus, TSHR A-subunit is thought to be preferentially immunogenic in GD (16), and also in animal GD models (17). Importantly, two portions in TSHR A-subunit (246–260 and 277–296) and another region in TSHR B-subunit (381–385) fold together to form a complex TSH-binding pocket (18).

GENETIC FACTORS IN GD

In research on twins, genetic factors were found to contribute 79% to the likelihood of having GD (19). In a Japanese nationwide study in 1999, 2.1–3.1% of hyperthyroidism seemed to be familial, and the relative risk of familial GD was increased from 19- to 42-fold (20). Tomer and Davies reported that 33% of siblings of AITD patients developed AITD themselves, and 56% of siblings of AITD patients produced thyroid autoantibodies,

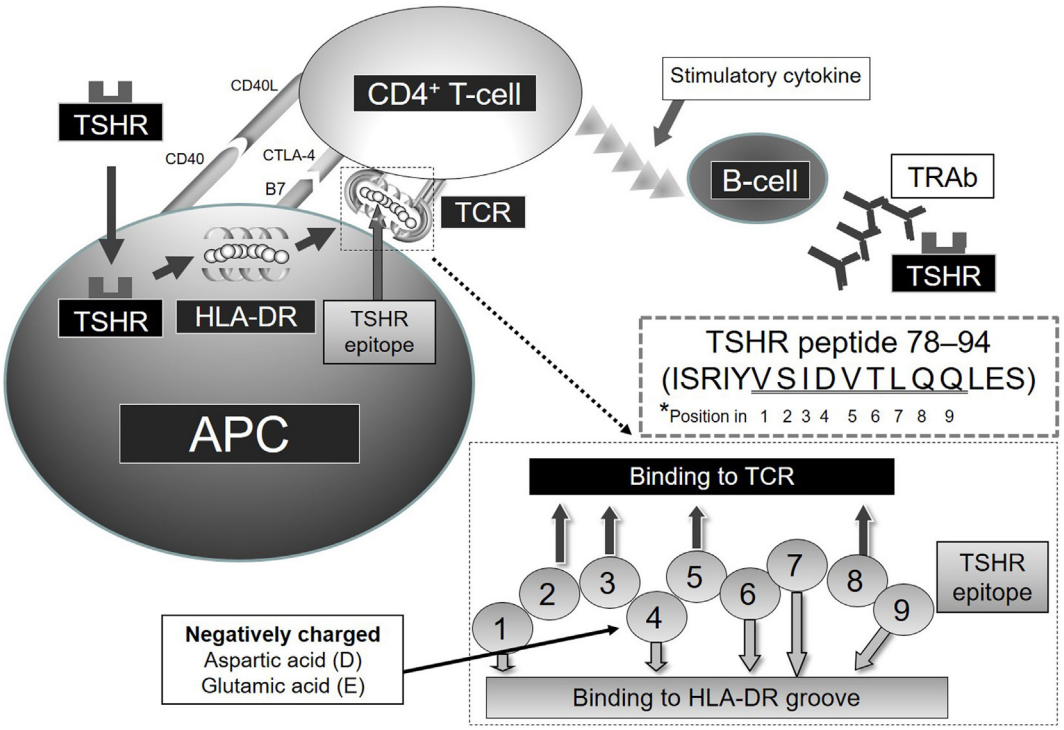


FIGURE 2 | Relation of TSHR and HLA-DR in GD *Note that amino acids of TSHR epitope 78–94 (underlined residues VSIDVTLQQ) are predicted to contact the HLA-DR-binding groove or TCR at positions 1–9, respectively.

also supporting a strong genetic influence on development of AITD (21). Genetic factors reported to predispose to GD include specific polymorphisms of HLA (3), CTLA-4 (4, 5), CD40 (6), protein tyrosine phosphatase-22 (PTPN22) (22), FOXP3, and CD25 (3). In addition, polymorphisms of TSHR (3, 4), Tg (3), interleukin-2 receptor alpha (IL2RA) (23), and Fc receptor-like3 (FCRL3) (24) were reported. Among these, HLA is a major genetic factor in AITD (3). The HLA locus is located on chromosome 6p21, and encodes (1) class I genes, such as HLA antigens A, B, and C, and (2) class II genes, such as HLA-DP, DQ, and DR genes (25). Inheritance of HLA-DRB1*03:01 (DR3) has been demonstrated to induce the highest susceptibility to GD in several ethnic groups (26, 27), and also in HT (3, 28). HLA-B8 was reported to be associated with GD in many studies (21). HLA-DQA1*05:01 was also reported to predispose to GD in Caucasians (26, 29). By contrast, HLA-DRB1*07:01 (DR7) was reported to be a protective allele for GD (30). The DR3 and DR7 alleles differ at the 74th amino acid in HLA-DRβ1, a critical residue in the binding pocket of the HLA-DR protein. The amino acid is arginine or glutamine, respectively. When DRB1*03:01 and DR7 alleles coexist, DR7 appears to suppress the susceptibility to GD conferred by DR3 (3). The HLA-genes have also been shown to be associated with GD in non-Caucasian populations, although the predisposing alleles are different from those observed in Caucasians. Chen et al. found that HLA-B*46:01, HLA-DPB1*05:01, HLA-DQB1*03:02, HLA-DRB1*15:01, and HLA-DRB1*16:02 were associated with GD in Taiwan (31). Recent studies in Japan have shown associations of GD with HLA-B*35:01, HLA-B*46:01, HLA-DRB1*14:03, and HLA-DPB1*05:01 (32). These authors reported that the protective allele, HLA-DRB1*13:02 overwhelms the GD-susceptibility of DP5 when they coexist. Many other gene associations have been reported. Vita et al. recently reported that certain HLA alleles are associated with stress-triggered GD and with clinical outcomes (33). The second most important gene polymorphism involves CTLA-4, which is expressed on activated T cells. It binds to B7 on the surface of APC to suppress T-cell-mediated immunity through co-suppressive signals (4).

One group has consistently reported association of TSHR gene polymorphisms with GD in Japanese (3, 4, 34). A Tg polymorphisms, in association with DR3, is also considered to relate to GD (3). Tomer et al. reported interaction between a Tg gene variant and DRB1-Arg 74 in predisposing to GD, increasing the odds ratio to more than 36 (28). Furthermore, they confirmed that TSHR, CTLA-4, and Tg genes are associated with GD in Italians (35). In an age-related aspect, Brown et al. identified novel susceptibility loci related to young age onset of GD (36). In a mouse model of GD, TRAb were genetically linked to both MHC-class I and Class II antigens (37).

Recently, Limbach et al. found hypermethylation of T-cell signaling genes and TSHR gene, suggesting dysregulation in T cell and TSHR signaling in GD patients (38). Stefan et al. also reported a genetic–epigenetic interaction involving a non-coding SNP in the TSHR gene that controls thymic TSHR gene expression and promotes escape of TSHR-reactive T cells from central tolerance, triggering GD (39).

RELATION OF TSHR-ECD AND HLA-DR

Shed TSHR-ECD protein is endocytosed into APCs and processed to TSHR-ECD peptides in lysosomes (**Figure 2**). These peptide epitopes bind to HLA-DR molecules, and subsequently the complex of HLA-DR and TSHR-ECD epitope is presented on APCs to CD4+ T cells. Aberrant expression of HLA-DR molecules was first considered as a trigger of GD (40), and later both TSHR and HLA-DR were found to be critical in the process of autoimmunity in GD (41). Recombinant human interferon (IFN)-α was reported to increase the expression of HLA-DR and TSHR on thyrocytes in GD subjects and not in controls (42). Shimojo et al. first reported that fibroblasts co-transfected with both human TSHR and MHC-class II could induce GD in mice (43). Lymphocytes infiltrating the thyroid in human TSHR A-subunit transgenic mice are involved in recognition of human TSHR A-subunit by T cells activated using adenovirus encoding the human TSHR (44). The complex of TSHR-ECD epitope presented on APC with MHC-class II and recognition by T cells appears to be necessary to initiate an immunogenic reaction.

We examined the binding affinity between TSHR-ECD epitopes and HLA-DR *in silico*, *in vitro*, and *in vivo* studies (7, 8, 45–47). Predicted binding affinities of TSHR-ECD peptides to epitope-binding groove in various HLA-DRs were examined using computer algorithms (7). These studies *in silico* and *in vitro* showed the priority of strong binders to HLA-DR in terms of immunogenicity. The peptide-binding groove in HLA-DR consists of nine amino acids. Amino acids in positions 1, 4, 6, 7, and 9 bind to HLA-DR and those in positions 2, 3, 5, and 8 face the TCR. We found that the amino acid position 4 of the amino acid sequence in the binding groove of HLA-DR is critical in determining binding affinity between the TSHR epitopes and HLA-DR (8). Positively charged Arginine in position 4 of the amino acid sequence in the binding motif of HLA-DRB1*03:01 appears also important (3, 8). TSHR-ECD epitopes with negatively charged D (aspartic acid) or E (glutamic acid) in position 4 of the binding motif bind more strongly to HLA-DR3 and are more stimulatory to GD patients' peripheral blood mononuclear cells and to splenocytes from HLA-DR3 mice immunized to TSHR-ECD (9). As a result, TSHR-ECD peptide 132–150 (GIFNTGLKMFPDLTKVYST) was identified *in silico*, *in vitro*, and in clinical assays as an important epitope in GD, and peptide 78–94 (ISRIYVSIDVTLQQLES) was also identified as an important epitope when additional peptides were synthesized and used for assay as candidate epitopes (7, 45) (**Figure 2**). The possible importance of TSHR epitopes having moderate binding affinities to HLA-DR3; residues 145–163, 158–176, 207–222, 248–263, 272–291, and 343–362 was also identified (7). These epitopes appear important in immunogenicity to TSHR due to their favored binding to HLA-DR3, thus increasing presentation to T cells (8, 45).

T- and B-cell responses to genetic immunization differ in DR3 and DR2 transgenic mice. Mice transgenic for HLA-DR3 were more prone to develop AITD than were HLA-DR2 transgenic mice (45, 48). Pichurin et al. reported that in DR3 transgenic mice immunized to adenovirus coding TSHR 1–289, TSHR peptide (142–161) that is close to one of the epitopes mentioned above appeared to be a major T-cell epitope (49). Other groups also defined the T-cell epitopes in development of GD (50–53). Martin et al. found TSHR peptides 52–71, 142–161, 202–221, and 247–266 to be frequently recognized by CD4+ T cells from patients with GD (52). Tandon et al. found that TSHR 146–165, 160–179, and 202–221 were relevant (53). A logical interpretation of the relation of epitope/DR binding to GD is that strong binding affinity to HLA-DR is related to high efficiency in antigen presentation (7). In fact, an exogenous antigen, such as *Yersinia* that possesses molecular mimicry with TSHR was reported to contribute to development of GD (54). Guarneri and Benvenga reported molecular mimicry between microbial and thyroid autoantigens, and proposed that microbial infection in predisposed subjects might initiate AITDs (55). Furthermore, they reported an *in silico* analysis for amino acid sequence homologies in HLA-DR-binding motifs between some microbial proteins and thyroid autoantigens (TSHR, Tg, and TPO). *Yersinia*, *Borrelia*, *Clostridium botulinum*, *Rickettsia*, and *Helicobacter pylori* were demonstrated to have molecular similarity to these thyroid autoantigens; thus, suggested to be associated with triggering AITD (56). They also reported a patient having HLA-DRB1*03:01 who developed GD possibly by rickettsial infection based on homology with hTSHR/HLA-DR*03:01 binding motif (57). Vita et al. found homology of tumor-associated antigens (NY-ESO-1) used as vaccines, with TSHR, Tg, and TPO in panels of HLA-class I- and class II-binding motifs (58). They concluded that AITD might be elicited by NY-ESO-1 vaccination.

Alternatively, peptides with high-binding affinities to HLA-DR molecules could lead to thymic deletion of the cognate T cells, while those peptides exhibiting moderate binding affinities could escape "negative selection" in the thymus and enter in the circulation and participate in autoimmune disease. Competition between low- and high-risk alleles for binding to TSHR peptides could also affect the development of GD. Due to a higher affinity for specific fragments, protective alleles might prevent binding and presentation of crucial epitopes by high-risk alleles. In addition, certain HLA alleles may not present important epitopes that induce TSHR antibodies. To date, prediction of binding of epitope to HLA-class II is possible as described above, and prediction of binding affinity between epitope and TCR is in development (59).

In mice, a splicing variant of mouse TSHR is related to GD. Endo and Kobayashi described "GD" in mice immunized to the TSHR gene lacking exon 5 (residues 132–157) (60). This observation suggested that exon 5 in TSHR may suppress GD progression, or antibody to residues in exon 5 may contribute to regulate immunity to TSHR. It also suggests that the TSHR-ECD peptide 132–150 (GIFNTGLKMFPDLTKVYST) noted above as a strong HLA binder may not be directly involved in pathogenesis of GD (7).

ROLE OF CYTOKINES/CHEMOKINES

For maturation of naïve CD4+ T cells (Th0) in the immunological network, activation of both the TCR and co-stimulatory molecules are necessary (61). APCs, as well as MHC-class II molecules, affect this process. This activation occurs by interaction among epitopes, APCs, and Th0 cells. Subsequently, local

cytokine regulation determines whether a Th0 cell will become a Th1 or Th2 cell. The presence of IL-12 and IFN-γ will activate signal transducer and transcription activator 4 (stat 4) and stat 1 signaling pathways, respectively, and promote Th1 cellular differentiation (61). For Th2 differentiation, IL-4 induces GATA3 through the stat-6 signaling pathway. Usually, Th1 cells produce IFN-γ, whereas Th2 cells secrete IL-4, 5, and 13. In GD, activated CD4+ T cells secrete cytokines/chemokines that stimulate B-cells to produce TRAb, and in turn hyperthyroidism occurs. Thus, a preferentially increased Th2/Th1 balance has been reported in GD (62). As IgG1 type of TSAb formation is seen initially in GD, an important role for Th1 was also reported (63). Novel cytokines and chemokines, such as CXCL10 were reported to be related to pathogenesis of GD (64). Increased serum levels of IL-21 (65) and decreased serum IL-7 (66) were also reported in GD. Intriguingly, an association of chromosome 5q23–q33 with AITD suggested an important role of clustered cytokines and other immune modulators encoded in this genetic locus (67). Another subset of Th cells, Th17 cells, was reported to play different roles in mice with different genetic backgrounds (68). Horie et al. genetically immunized NOD-H2(h4) or BALB/c mice with TSHR A-subunit, and found that IL-17 was indispensable for development of Graves' hyperthyroidism in non-GD-susceptible NOD-H2(h4), but not in GD-susceptible BALB/c mice (68).

B-CELL EPITOPE AND TRAb IN GD

In addition to the T-cell epitopes described above, numerous studies have identified B-cell epitopes of TSHR-ECD in GD. The structure of the Fab fragment of IgG determines the binding affinity to TSHR. TRAb consists of (1) TSAb: thyroid-stimulating antibody, (2) TSBAb: thyroid-stimulation blocking antibody, and (3) neutral TRAb (1, 9, 69). (1) Studies suggest that TSAbs interact with the N-terminal region of the TSHR and transduce a signal through binding sites different from the TSH-binding site (70). R38 in TSHR is the major N-terminal contact residue of TSAb, M22 (71). The TSAb epitopes require involvement of the highly conformational N-terminus of the A-subunit (72). TSAb also preferentially recognize the free A-subunit in animal studies (73). Binding of anti TSHR antibodies to the amino-terminal end of the ECD was confirmed in DR3 transgenic mice (46). TSBAb, usually seen in patients with primary myxedema, recognize the C-terminal region of TSHR-ECD. The antibodies that bound to TSHR residue 381–385 blocked TSHR stimulation by TSH (74).

In contrast to the other glycoprotein hormone receptors, TSHR has ligand-independent (constitutive) activity. Chen et al. found that monoclonal antibody, CS-17, significantly reduces this constitutive activity. This antibody, thus, was considered as "inverse agonist," but binds to N-terminus of TSHR-ECD, residues 260–289 (75). Rees Smith et al. also showed that antibodies of both stimulating and blocking types bind well to the TSHR (residues 22–260) (76). Neutral TRAb have a linear epitope confined to the cleaved region of TSHR (residues 316–366) (77). By contrast, Hamidi et al. investigated properties of non-stimulatory murine monoclonal antibody, 3BD10. The linear epitope locates in TSHR (residues 31–41) (78). Clinically, Soliman et al. reported that simultaneous recognition of peptides TSHR 158–176 and 248–263 is important for the development of GD (79). In contrast to GD, the functional epitopes of TRAb in HT patients were reported to be uniquely different from those observed in GD (80). A female patient with HT had a blocking type TBII and a weak TSAb. Her blocking type TBII was uniquely reactive with the N-terminal, rather than C-terminal of TSHR-ECD. In addition, her TSAb epitope did not appear to be present solely on the N- or C-terminus of the TSHR-ECD (although the functional epitopes of most TSAb are known to involve the N-terminal region of the receptor) (80). Multimers of TSHR, not monomers, may be required for the maturation of TRAb (81). While little interest is directed to MHC-class II in the development of B-cell epitope in GD, T-cell activation through MHC-class II is indispensable for maturation of TRAb-producing B-cell.

Measurement of TRAb plays an important role in clinical practice. A meta-analysis showed that the overall sensitivity and specificity of the second- and third-generation TRAb assays in GD are 97.1 and 97.4%, and 98.3 and 99.2%, respectively. The likelihood of TRAb-positive individuals to have GD is 1367- to 3420-fold greater compared to that of a TRAb-negative person (82).

CENTRAL AND PERIPHERAL TOLERANCE

In central tolerance, immature T cells with high affinity for autoantigen-derived peptides are deleted in the thymus (7). Regulatory T cells (Tregs) play an important role in suppressing immunogenic T cells in the periphery (peripheral tolerance) (83). Dysfunction of central tolerance in the thymus or Tregs would allow onset of GD. Thyroid autoantigen expression of TSHR, TPO, and Tg in the thymus was not significantly different in different mouse strains (84), suggesting that not only thyroid autoantigen presentation with various MHC molecules but also co-activators or other factors must control self-tolerance. In mouse studies, Tregs are reported not to be involved in TSHR self-tolerance (2). Tregs control the balance between GD and HT (2). Treg numbers in human GD were reported not to be decreased, but Treg function was suggested to be impaired (83, 85). Recent articles further support this Treg functional impairment in several types of CD4+ Treg cells (Foxp3+, CD69+, Tr1) (86).

EPITOPE SPREADING DURING PROGRESSION OF GD

In the course of pathogenic amplification of immunogenic T- and B-cells in GD, "epitope spreading" is frequently seen (50, 69, 87). Intra molecular (TSHR) (69, 88) and inter molecular (Tg, TPO) (2) epitope spreading are observed. The mechanism may relate to developing immunity to host TSHR, and epitope spreading along this antigen (human TSHR to mouse TSHR) (17). Possible reason for T-cell epitope spreading may be the heterogeneity in recognizing thyroid autoantigen (89), or re-arrangements of TCR gene (90). IgG VH gene re-arrangement is known to be associated with B-cell epitope spreading (1, 91). Segundo et al. reported that the occurrence of two distinct types of Thyroid-infiltrating B-lymphocytes. Type 1 B-lymphocytes showed features of marginal zone B-cells, and type 2 B-lymphocytes exhibited a

phenotype of germinal center B-cells. They suggested that type 2 might be associated with high titers of TPOAb and not anti-TSHR antibody (92). The role of thyroid-infiltrating B cell in TSHR-related B-cell epitope spreading is yet clear.

PERSPECTIVES ON TSHR-SPECIFIC TREATMENTS

A novel small molecular TSHR antagonist has been demonstrated to be effective in animal studies as a TSHR-specific treatment for GD (93). TSHR epitope-specific treatments using mutated TSHR peptides were reported to suppress immunogenic reaction of TSHR-ECD in HLA-DR3 transgenic mice immunized to TSHR-ECD protein (46). Peptides in HLA-DR-binding positions 2, 3, 5, and 8 are assumed to be outward facing to stimulate the TCR (8). Therefore, a mutant TSHR peptide was constructed in which the contact of peptide to TCR would be attenuated (46). TSHR peptide 78–94: ISRIYVSID**V**TL**Q**QLES was mutated to TSHR peptide 37m: ISRIYVSID**A**TL**S**QLES, in which DR3-binding motif position 5 was mutated V > A, and position 8 Q > S. 37m was predicted to bind to HLA-DR3, but not bind strongly to TCRs. Both antibody titers to TSHR peptide 78–94, and reaction of splenocytes to TSHR peptide 78–94, were significantly reduced in mice immunized to TSHR peptide 78–94 plus 37m, compared to mice immunized to TSHR peptide 78–94 alone.

The goal of inducing self-tolerance to prevent AITD will require accurate prediction of at-risk individuals together with an antigen-specific therapeutic approach. A transgenic mouse strain having spontaneous TRAb production was developed, and offers further opportunities for investigation of GD *in vivo* (94, 95). As a B-cell-targeted therapy, anti CD20 antibody was reported to be effective for thyroid associated orbitopathy (96). In addition, as well as the acquired immunity described above, innate immunity was suggested to be involved with development of GD (97, 98). Pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), and iodide effects on gene expression were reported to be related to innate immune responses (97). The expression of toll-like receptor 4 in thyroid cells may be associated with development of AITDs (98). Thus, specific treatment targeted to innate immunity might be hopeful.

CONCLUSION

In the recent years, remarkable progression of research in the mechanism underlying GD was seen. In addition to the function and conformation of TSHR, its binding interaction to HLA-class II molecules and presentation to T cells have been investigated. The relation of TSHR and HLA in terms of TSHR epitope presentation is crucial in development of GD. Numerous studies to identify T- and B-cell epitopes have also demonstrated, including (1) *in silico*, (2) *in vitro*, (3) *in vivo*, and (4) clinical experiments. Dysfunction of central and peripheral tolerance could contribute to development of GD. Although key ideas have been proposed, further investigations are warranted to elucidate precise immunological systems in GD and to establish TSHR epitope-specific treatment.

AUTHOR CONTRIBUTIONS

All authors were involved in the preparation and writing of the manuscript.

FUNDING

This work was partially supported by Grants-in-Aid for Scientific Research 26461385, and Takeda Science foundation.

REFERENCES

1. Akamizu T, Mori T, Nakao K. Pathogenesis of Graves' disease: molecular analysis of anti-thyrotropin receptor antibodies. *Endocr J* (1997) 44:633–46. doi:10.1507/endocrj.44.633
2. McLachlan SM, Rapoport B. Breaking tolerance to thyroid antigens: changing concepts in thyroid autoimmunity. *Endocr Rev* (2014) 35:59. doi:10.1210/er.2013-1055
3. Lee HJ, Li CW, Hammerstad SS, Stefan M, Tomer Y. Immunogenetics of autoimmune thyroid diseases: a comprehensive review. *J Autoimmun* (2015) 64:82. doi:10.1016/j.jaut.2015.07.009
4. Akamizu T, Sale MM, Rich SS, Hiratani H, Noh JY, Kanamoto N, et al. Association of autoimmune thyroid disease with microsatellite markers for the thyrotropin receptor gene and CTLA-4 in Japanese patients. *Thyroid* (2000) 10:851–8. doi:10.1089/thy.2000.10.851
5. Yanagawa T, Hidaka Y, Guimaraes V, Soliman M, DeGroot LJ. CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population. *J Clin Endocrinol Metab* (1995) 80:41–5. doi:10.1210/jc.80.1.41
6. Kurylowicz A, Kula D, Ploski R, Skorka A, Jurecka-Lubieniecka B, Zebracka J, et al. Association of CD40 gene polymorphism (C-1T) with susceptibility and phenotype of Graves' disease. *Thyroid* (2005) 15:1119–24. doi:10.1089/thy.2005.15.1119
7. Inaba H, Martin W, De Groot AS, Qin S, De Groot LJ. Thyrotropin receptor epitopes and their relation to histocompatibility leukocyte antigen-DR molecules in Graves' disease. *J Clin Endocrinol Metab* (2006) 91:2286–94. doi:10.1210/jc.2005-2537
8. Inaba H, Martin W, Ardito M, De Groot AS, De Groot LJ. The role of glutamic or aspartic acid in position four of the epitope binding motif and thyrotropin receptor-extracellular domain epitope selection in Graves' disease. *J Clin Endocrinol Metab* (2010) 95:2909–16. doi:10.1210/jc.2009-2393
9. Akamizu T. Antithyrotropin receptor antibody: an update. *Thyroid* (2001) 11:1123. doi:10.1089/10507250152740966
10. Akamizu T, Ikuyama S, Saji M, Kosugi S, Kozak C, McBride OW, et al. Cloning, chromosomal assignment, and regulation of the rat thyrotropin receptor: expression of the gene is regulated by thyrotropin, agents that increase cAMP levels, and thyroid autoantibodies. *Proc Natl Acad Sci U S A* (1990) 87:5677–81. doi:10.1073/pnas.87.15.5677
11. Kleinau G, Neumann S, Grüters A, Krude H, Biebermann H. Novel insights on thyroid-stimulating hormone receptor signal transduction. *Endocr Rev* (2013) 34:691–724. doi:10.1210/er.2012-1072
12. Brüser A, Schulz A, Rothemund S, Ricken A, Calebiro D, Kleinau G, et al. The activation mechanism of glycoprotein hormone receptors with implications in the cause and therapy of endocrine diseases. *J Biol Chem* (2016) 8(291):508–20. doi:10.1074/jbc.M115.701102
13. Schaarschmidt J, Nagel MB, Huth S, Jaeschke H, Moretti R, Hintze V, et al. Rearrangement of the extracellular domain/extracellular loop 1 interface is critical for thyrotropin receptor activation. *J Biol Chem* (2016) 291(27):14095–108. doi:10.1074/jbc.M115.709659
14. Rapoport B, McLachlan SM. TSH receptor cleavage into subunits and shedding of the A-subunit; a molecular and clinical perspective. *Endocr Rev* (2016) 37:114–34. doi:10.1210/er.2015-1098

15. Wadsworth HL, Chazenbalk GD, Nagayama Y, Russo D, Rapoport B. An insertion in the human thyrotropin receptor critical for high affinity hormone binding. *Science* (1990) 249:1423–5. doi:10.1126/science.2169649
16. Mizutori Y, Chen CR, Latrofa F, McLachlan SM, Rapoport B. Evidence that shed thyrotropin receptor A subunits drive affinity maturation of autoantibodies causing Graves' disease. *J Clin Endocrinol Metab* (2009) 94:927–35. doi:10.1210/jc.2008-2134
17. McLachlan SM, Nagayama Y, Rapoport B. Insight into Graves' hyperthyroidism from animal models. *Endocr Rev* (2005) 26:800. doi:10.1210/er.2004-0023
18. Jeffreys J, Depraetere H, Sanders J, Oda Y, Evans M, Kiddie A, et al. Characterization of the thyrotropin binding pocket. *Thyroid* (2002) 12:1051–61. doi:10.1089/105072502321085144
19. Brix TH, Kyvik KO, Christensen K, Hegedüs L. Evidence for a major role of heredity in Graves' disease: a population-based study of two Danish twin cohorts. *J Clin Endocrinol Metab* (2001) 86:930–4. doi:10.1210/jc.86.2.930
20. Akamizu T, Nakamura Y, Tamaoki A, Inaba Y, Amino N, Seino Y. Prevalence and clinico-epidemiology of familial Graves' disease in Japan based on nationwide epidemiologic survey in 2001. *Endocr J* (2003) 50:429–36. doi:10.1507/endocrj.50.429
21. Tomer Y, Davies TF. Searching for the autoimmune thyroid disease susceptibility genes: from gene mapping to gene function. *Endocr Rev* (2003) 24:694–717. doi:10.1210/er.2002-0030
22. Heward JM, Brand OJ, Barrett JC, Carr-Smith JD, Franklyn JA, Gough SC. Association of PTPN22 haplotypes with Graves' disease. *J Clin Endocrinol Metab* (2007) 92:685–90. doi:10.1210/jc.2006-2064
23. Brand OJ, Lowe CE, Heward JM, Franklyn JA, Cooper JD, Todd JA, et al. Association of the interleukin-2 receptor alpha (IL-2Ralpha)/CD25 gene region with Graves' disease using a multilocus test and tag SNPs. *Clin Endocrinol (Oxf)* (2007) 66:508–12. doi:10.1111/i.1365-2265.2007.02752.x
24. Kochi Y, Yamada R, Suzuki A, Harley JB, Shirasawa S, Sawada T, et al. A functional variant in FCRL3, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. *Nat Genet* (2005) 37:478–85. doi:10.1038/ng1540
25. Nelson JL, Hansen JA. Autoimmune diseases and HLA. *Crit Rev Immunol* (1990) 10:307–28.
26. Maciel LM, Rodrigues SS, Dibbern RS, Navarro PA, Donadi EA. Association of the HLA-DRB1*0301 and HLA-DQA1*0501 alleles with Graves' disease in a population representing the gene contribution from several ethnic backgrounds. *Thyroid* (2001) 11:31–5. doi:10.1089/10507250150500630
27. Ahn S, Choi HB, Kim TG. HLA and disease associations in Koreans. *Immune Netw* (2011) 11:324–35. doi:10.4110/in.2011.11.6.324
28. Jacobson EM, Huber A, Tomer Y. The HLA gene complex in thyroid autoimmunity: from epidemiology to etiology. *J Autoimmun* (2008) 30:58–62. doi:10.1016/j.jaut.2007.11.010
29. Yanagawa T, Mangklabruks A, Chang YB, Okamoto Y, Fisfalen ME, Curran PG, et al. Human histocompatibility leukocyte antigen-DQA1*0501 allele associated with genetic susceptibility to Graves' disease in a Caucasian population. *J Clin Endocrinol Metab* (1993) 76:1569–74. doi:10.1210/jc.76.6.1569
30. Zeitlin AA, Heward JM, Newby PR, Carr-Smith JD, Franklyn JA, Gough SC, et al. Analysis of HLA class II genes in Hashimoto's thyroiditis reveals differences compared to Graves' disease. *Genes Immun* (2008) 9:358–63. doi:10.1038/gene.2008.26
31. Chen PL, Fann CS, Chu CC, Chang CC, Chang SW, Hsieh HY, et al. Comprehensive genotyping in two homogeneous Graves' disease samples reveals major and novel HLA association alleles. *PLoS One* (2011) 28:e16635. doi:10.1371/journal.pone.0016635
32. Ueda S, Oryoji D, Yamamoto K, Noh JY, Okamura K, Noda M, et al. Identification of independent susceptible and protective HLA alleles in Japanese autoimmune thyroid disease and their epistasis. *J Clin Endocrinol Metab* (2014) 99:E379–83. doi:10.1210/jc.2013-2841
33. Vita R, Lapa D, Trimarchi F, Vita G, Fallahi P, Antonelli A, et al. Certain HLA alleles are associated with stress-triggered Graves' disease and influence its course. *Endocrine* (2016). doi:10.1007/s12020-016-0909-6
34. Hiratani H, Bowden DW, Ikegami S, Shirasawa S, Shimizu A, Iwatani Y, et al. Multiple SNPs in intron 7 of thyrotropin receptor are associated with Graves' disease. *J Clin Endocrinol Metab* (2005) 90:2898–903. doi:10.1210/jc.2004-2148
35. Lombardi A, Menconi F, Greenberg D, Concepcion E, Leo M, Rocchi R, et al. Dissecting the genetic susceptibility to Graves' disease in a cohort of patients of Italian origin. *Front Endocrinol* (2016) 7:21. doi:10.3389/fendo.2016.00021
36. Brown RS, Lombardi A, Hasham A, Greenberg DA, Gordon J, Concepcion E, et al. Genetic analysis in young-age-of-onset Graves' disease reveals new susceptibility loci. *J Clin Endocrinol Metab* (2014) 99:E1387–91. doi:10.1210/jc.2013-4358
37. McLachlan SM, Aliesky HA, Chen CR, Williams RW, Rapoport B. Exceptional hyperthyroidism and a role for both major histocompatibility class I and class II genes in a murine model of Graves' disease. *PLoS One* (2011) 6:e21378. doi:10.1371/journal.pone.0021378
38. Limbach M, Saare M, Tserel L, Kisand K, Eglit T, Sauer S, et al. Epigenetic profiling in CD4+ and CD8+ T cells from Graves' disease patients reveals changes in genes associated with T cell receptor signaling. *J Autoimmun* (2016) 67:46–56. doi:10.1016/j.jaut.2015.09.006
39. Stefan M, Wei C, Lombardi A, Li CW, Concepcion ES, Inabnet WB, et al. Genetic-epigenetic dysregulation of thymic TSH receptor gene expression triggers thyroid autoimmunity. *Proc Natl Acad Sci U S A* (2014) 111:12562–7. doi:10.1073/pnas.1408821111
40. Hanafusa T, Pujol-Borrell R, Chiovato L, Russell RC, Doniach D, Bottazzo GF. Aberrant expression of HLA-DR antigen on thyrocytes in Graves' disease: relevance for autoimmunity. *Lancet* (1983) 12(2):1111–5. doi:10.1016/S0140-6736(83)90628-1
41. Li YS, Kanamoto N, Hataya Y, Moriyama K, Hiratani H, Nakao K, et al. Transgenic mice producing major histocompatibility complex class II molecules on thyroid cells do not develop apparent autoimmune thyroid diseases. *Endocrinology* (2004) 145:2524–30. doi:10.1210/en.2003-1654
42. Kuang M, Wang S, Wu M, Ning G, Yao Z, Li L. Expression of IFNalpha-inducible genes and modulation of HLA-DR and thyroid stimulating hormone receptors in Graves' disease. *Mol Cell Endocrinol* (2010) 5(319):23–9. doi:10.1016/j.mce.2009.12.006
43. Shimojo N, Kohno Y, Yamaguchi K, Kikuoka S, Hoshioka A, Niimi H, et al. Induction of Graves-like disease in mice by immunization with fibroblasts transfected with the thyrotropin receptor and a class II molecule. *Proc Natl Acad Sci U S A* (1996) 1(93):11074–9. doi:10.1073/pnas.93.20.11074
44. Mizutori Y, Nagayama Y, Flower D, Misharin A, Aliesky HA, Rapoport B, et al. Role of the transgenic human thyrotropin receptor A-subunit in thyroiditis induced by A-subunit immunization and regulatory T cell depletion. *Clin Exp Immunol* (2008) 154:305–15. doi:10.1111/j.1365-2249.2008.03769.x
45. Inaba H, Pan D, Shin YH, Martin W, Buchman G, De Groot LJ. Immune response of mice transgenic for human histocompatibility leukocyte Antigen-DR to human thyrotropin receptor-extracellular domain. *Thyroid* (2009) 19:1271–80. doi:10.1089/thy.2008.0349
46. Inaba H, Moise L, Martin W, De Groot AS, Desrosiers J, Tassone R, et al. Epitope recognition in HLA-DR3 transgenic mice immunized to TSH-R protein or peptides. *Endocrinology* (2013) 154:2234–43. doi:10.1210/en.2013-1033
47. Sawai Y, DeGroot LJ. Binding of human thyrotropin receptor peptides to a Graves' disease-predisposing human leukocyte antigen class II molecule. *J Clin Endocrinol Metab* (2000) 85:1176–9. doi:10.1210/jc.85.3.1176
48. Kong YC, Lomo LC, Motte RW, Giraldo AA, Baisch J, Strauss G, et al. HLA-DRB1 polymorphism determines susceptibility to autoimmune thyroiditis in transgenic mice: definitive association with HLA-DRB1*0301 (DR3) gene. *J Exp Med* (1996) 1(184):1167–72. doi:10.1084/jem.184.3.1167
49. Pichurin P, Pham N, David CS, Rapoport B, McLachlan SM. HLA-DR3 transgenic mice immunized with adenovirus encoding the thyrotropin receptor: T cell epitopes and functional analysis of the CD40 Graves' polymorphism. *Thyroid* (2006) 16:1221–7. doi:10.1089/thy.2006.16.1221
50. Sugawa H, Akamizu T, Kosugi S, Ueda Y, Ohta C, Okuda J, et al. Presence of heterogeneous thyroid-stimulating antibodies in sera from individual Graves' patients as shown by synthesized thyrotropin receptor peptide application: evidence showing two independent epitopes and a possible recognition of two epitopic regions by one antibody molecule. *Eur J Endocrinol* (1995) 133:283–93.
51. Soliman M, Kaplan E, Yanagawa T, Hidaka Y, Fisfalen ME, DeGroot LJ. T-cells recognize multiple epitopes in the human thyrotropin receptor extracellular domain. *J Clin Endocrinol Metab* (1995) 80:905–14. doi:10.1210/jc.80.3.905
52. Martin A, Nakashima M, Zhou A, Aroson D, Werner AJ, Davies TF. Detection of major T-cell epitopes on human thyroid stimulating hormone

receptorbyoverridingimmuneheterogeneityinpatientswithGraves'disease. *J Clin Endocrinol Metab* (1997) 82:3361–6. doi:10.1210/jc.82.10.3361
53. Tandon N, Freeman MA, Weetman AP. Responses to synthetic TSH receptor peptides in Graves' disease. *Clin Exp Immunol* (1992) 89:468–73. doi:10.1111/j.1365-2249.1992.tb06982.x
54. Hargreaves CE, Grasso M, Hampe CS, Stenkova A, Atkinson S, Joshua GW, et al. *Yersinia enterocolitica* provides the link between thyroid-stimulating antibodies and their germline counterparts in Graves' disease. *J Immunol* (2013) 1(190):5373–81. doi:10.4049/jimmunol.1203412
55. Guarneri F, Benvenga S. Environmental factors and genetic background that interact to cause autoimmune thyroid disease. *Curr Opin Endocrinol Diabetes Obes* (2007) 14:398–409. doi:10.1097/MED.0b013e3282ef1c48
56. Benvenga S, Guarneri F. Molecular mimicry and autoimmune thyroid disease. *Rev Endocr Metab Disord* (2016). doi:10.1007/s11154-016-9363-2
57. Marangou A, Guarneri F, Benvenga S. Graves' disease precipitated by rickettsial infection. *Endocrine* (2015) 50:828–9. doi:10.1007/s12020-015-0767-7
58. Vita R, Guarneri F, Agah R, Benvenga S. Autoimmune thyroid disease elicited by NY-ESO-1 vaccination. *Thyroid* (2014) 24:390–4. doi:10.1089/thy.2013.0170
59. Moise L, Beseme S, Tassone R, Liu R, Kibria F, Terry F, et al. T cell epitope redundancy: cross-conservation of the TCR face between pathogens and self and its implications for vaccines and autoimmunity. *Expert Rev Vaccines* (2016) 15:607–17. doi:10.1586/14760584.2016.1123098
60. Endo T, Kobayashi T. Immunization of mice with a newly identified thyroid-stimulating hormone receptor splice variant induces Graves'-like disease. *J Autoimmun* (2013) 43:18–25. doi:10.1016/j.jaut.2013.02.004
61. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood* (2008) 1(112):1557–69. doi:10.1182/blood-2008-05-078154
62. Phenekos C, Vryonidou A, Gritzapis AD, Baxevanis CN, Goula M, Papamichail M. Th1 and Th2 serum cytokine profiles characterize patients with Hashimoto's thyroiditis (Th1) and Graves' disease (Th2). *Neuroimmunomodulation* (2004) 11:209–13. doi:10.1159/000078438
63. Rapoport B, McLachlan SM. Graves' hyperthyroidism is antibody-mediated but is predominantly a Th1-type cytokine disease. *J Clin Endocrinol Metab* (2014) 99:4060–1. doi:10.1210/jc.2014-3011
64. Antonelli A, Ferrari SM, Corrado A, Di Domenicantonio A, Fallahi P. Autoimmune thyroid disorders. *Autoimmun Rev* (2015) 14:174. doi:10.1016/j.autrev.2014.10.016
65. Zhang J, Zeng H, Ren M, Yan H, Xu M, Feng Z, et al. Interleukin-21 is associated with disease activity in patients with Graves' disease. *Endocrine* (2014) 46:539–48. doi:10.1007/s12020-013-0105-x
66. Motylewska E, Nieć M, Siejka A, Komorowski J, Ławnicka H, Świętosławski J, et al. Decreased serum level of IL-7 in patients with active Graves' disease. *Cytokine* (2015) 75:373–9. doi:10.1016/j.cyto.2015.04.020
67. Akamizu T, Hiratani H, Ikegami S, Rich SS, Bowden DW. Association study of autoimmune thyroid disease at 5q23-q33 in Japanese patients. *J Hum Genet* (2003) 48:236–42. doi:10.1007/s10038-003-0017-3
68. Horie I, Abiru N, Saitoh O, Ichikawa T, Iwakura Y, Eguchi K, et al. Distinct role of T helper Type 17 immune response for Graves' hyperthyroidism in mice with different genetic backgrounds. *Autoimmunity* (2011) 44:159–65. doi:10.3109/08916931003777247
69. Akamizu T, Kohn LD, Mori T. Molecular studies on thyrotropin (TSH) receptor and anti-TSH receptor antibodies. *Endocr J* (1995) 42:617–27. doi:10.1507/endocrj.42.617
70. Akamizu T, Moriyama K, Miura M, Saijo M, Matsuda F, Nakao K. Characterization of recombinant monoclonal antithyrotropin receptor antibodies (TSHRAbs) derived from lymphocytes of patients with Graves' disease: epitope and binding study of two stimulatory TSHRAbs. *Endocrinology* (1999) 140:1594–601. doi:10.1210/endo.140.4.6664
71. Smith BR, Sanders J, Furmaniak J. TSH receptor antibodies. *Thyroid* (2007) 17:923–38. doi:10.1089/thy.2007.0239
72. Schwarz-Lauer L, Pichurin PN, Chen CR, Nagayama Y, Paras C, Morris JC, et al. The cysteine-rich amino terminus of the thyrotropin receptor is the immunodominant linear antibody epitope in mice immunized using naked deoxyribonucleic acid or adenovirus vectors. *Endocrinology* (2003) 144:1718–25. doi:10.1210/en.2002-0069
73. Chen CR, Hubbard PA, Salazar LM, McLachlan SM, Murali R, Rapoport B. Crystal structure of a TSH receptor monoclonal antibody: insight into Graves' disease pathogenesis. *Mol Endocrinol* (2015) 29:99–107. doi:10.1210/me.2014-1257
74. Oda Y, Sanders J, Evans M, Kiddie A, Munkley A, James C, et al. Epitope analysis of the human thyrotropin (TSH) receptor using monoclonal antibodies. *Thyroid* (2000) 10:1051–9. doi:10.1089/thy.2000.10.1051
75. Chen CR, McLachlan SM, Rapoport B. Suppression of thyrotropin receptor constitutive activity by a monoclonal antibody with inverse agonist activity. *Endocrinology* (2007) 148:2375–82. doi:10.1210/en.2006-1754
76. Rees Smith B, Sanders J, Evans M, Tagami T, Furmaniak J. TSH receptor – autoantibody interactions. *Horm Metab Res* (2009) 41:448–55. doi:10.1055/s-0029-1220913
77. Morshed SA, Ando T, Latif R, Davies TF. Neutral antibodies to the TSH receptor are present in Graves' disease and regulate selective signaling cascades. *Endocrinology* (2010) 151:5537–49. doi:10.1210/en.2010-0424
78. Hamidi S, Chen CR, Murali R, McLachlan SM, Rapoport B. Probing structural variability at the N terminus of the TSH receptor with a murine monoclonal antibody that distinguishes between two receptor conformational forms. *Endocrinology* (2013) 154:562–71. doi:10.1210/en.2012-1822
79. Soliman M, Kaplan E, Abdel-Latif A, Scherberg N, DeGroot LJ. Does thyroidectomy, radioactive iodine therapy, or antithyroid drug treatment alter reactivity of patients' T cells to epitopes of thyrotropin receptor in autoimmune thyroid diseases? *J Clin Endocrinol Metab* (1995) 80:2312–21. doi:10.1210/jcem.80.8.7543112
80. Akamizu T, Kohn LD, Hiratani H, Saijo M, Tahara K, Nakao K. Hashimoto's thyroiditis with heterogeneous antithyrotropin receptor antibodies: unique epitopes may contribute to the regulation of thyroid function by the antibodies. *J Clin Endocrinol Metab* (2000) 85:2116–21. doi:10.1210/jc.85.6.2116
81. Rapoport B, Aliesky HA, Chen CR, McLachlan SM. Evidence that TSH receptor A-subunit multimers, not monomers, drive antibody affinity maturation in Graves' disease. *J Clin Endocrinol Metab* (2015) 100:E871–5. doi:10.1210/jc.2015-1528
82. Tozzoli R, Bagnasco M, Giavarina D, Bizzaro N. TSH receptor autoantibody immunoassay in patients with Graves' disease: improvement of diagnostic accuracy over different generations of methods. Systematic review and meta-analysis. *Autoimmun Rev* (2012) 12:107–13. doi:10.1016/j.autrev.2012.07.003
83. Glick AB, Wodzinski A, Fu P, Levine AD, Wald DN. Impairment of regulatory T-cell function in autoimmune thyroid disease. *Thyroid* (2013) 23:871–8. doi:10.1089/thy.2012.0514
84. Misharin AV, Rapoport B, McLachlan SM. Thyroid antigens, not central tolerance, control responses to immunization in BALB/c versus C57BL/6 mice. *Thyroid* (2009) 19:503–9. doi:10.1089/thy.2008.0420
85. Pan D, Shin YH, Gopalakrishnan G, Hennessey J, De Groot LJ. Regulatory T cells in Graves' disease. *Clin Endocrinol (Oxf)* (2009) 71:587–93. doi:10.1111/j.1365-2265.2009.03544.x
86. González-Amaro R, Marazuela M. T regulatory (Treg) and T helper 17 (Th17) lymphocytes in thyroid autoimmunity. *Endocrine* (2016) 52:30–8. doi:10.1007/s12020-015-0759-7
87. Akamizu T, Ueda Y, Hua L, Okuda J, Mori T. Establishment and characterization of an antihuman thyrotropin (TSH) receptor-specific CD4+ T cell line from a patient with Graves' disease: evidence for multiple T cell epitopes on the TSH receptor including the transmembrane domain. *Thyroid* (1995) 5:259–64. doi:10.1089/thy.1995.5.259
88. Vlase H, Nakashima M, Graves PN, Tomer Y, Morris JC, Davies TF. Defining the major antibody epitopes on the human thyrotropin receptor in immunized mice: evidence for intramolecular epitope spreading. *Endocrinology* (1995) 136:4415–23. doi:10.1210/endo.136.10.7664661
89. Dayan CM, Londei M, Corcoran AE, Grubeck-Loebenstein B, James RF, Rapoport B, et al. Autoantigen recognition by thyroid-infiltrating T cells in Graves disease. *Proc Natl Acad Sci U S A* (1991) 15(88):7415–9. doi:10.1073/pnas.88.16.7415
90. Martin A, Barbesino G, Davies TF. T-cell receptors and autoimmune thyroid disease – signposts for T-cell-antigen driven diseases. *Int Rev Immunol* (1999) 18:111–40. doi:10.3109/08830189909043021
91. Tonegawa S. Somatic generation of antibody diversity. *Nature* (1983) 302:575–81. doi:10.1038/302575a0
92. Segundo C, Rodríguez C, Aguilar M, García-Poley A, Gavilán I, Bellas C, et al. Differences in thyroid-infiltrating B lymphocytes in patients with Graves'

disease: relationship to autoantibody detection. *Thyroid* (2004) 14:337–44. doi:10.1089/105072504774193159
93. Neumann S, Eliseeva E, McCoy JG, Napolitano G, Giuliani C, Monaco F, et al. A new small-molecule antagonist inhibits Graves' disease antibody activation of the TSH receptor. *J Clin Endocrinol Metab* (2011) 96:548–54. doi:10.1210/jc.2010-1935
94. Kim-Saijo M, Akamizu T, Ikuta K, Iida Y, Ohmori K, Matsubara K, et al. Generation of a transgenic animal model of hyperthyroid Graves' disease. *Eur J Immunol* (2003) 33:2531–8. doi:10.1002/eji.200324255
95. Rapoport B, Aliesky HA, Banuelos B, Chen CR, McLachlan SM. A unique mouse strain that develops spontaneous, iodine-accelerated, pathogenic antibodies to the human thyrotrophin receptor. *J Immunol* (2015) 1(194):4154–61. doi:10.4049/jimmunol.1500126
96. Stan MN, Garrity JA, Carranza Leon BG, Prabin T, Bradley EA, Bahn RS. Randomized controlled trial of rituximab in patients with Graves' orbitopathy. *J Clin Endocrinol Metab* (2015) 100:432. doi:10.1210/jc.2014-2572
97. Kawashima A, Yamazaki K, Hara T, Akama T, Yoshihara A, Sue M, et al. Demonstration of innate immune responses in the thyroid gland: potential to sense danger and a possible trigger for autoimmune reactions. *Thyroid* (2013) 23:477–87. doi:10.1089/thy.2011.0480
98. Nicola JP, Vélez ML, Lucero AM, Fozzatti L, Pellizas CG, Masini-Repiso AM. Functional toll-like receptor 4 conferring lipopolysaccharide responsiveness is expressed in thyroid cells. *Endocrinology* (2009) 150:500–8. doi:10.1210/en.2008-0345

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 14 November 2016
doi: 10.3389/fendo.2016.00144

Excessive Cytosolic DNA Fragments as a Potential Trigger of Graves' Disease: An Encrypted Message Sent by Animal Models

*Yuqian Luo[1], Aya Yoshihara[1,2], Kenzaburo Oda[1,3], Yuko Ishido[1] and Koichi Suzuki[1]**

[1] Department of Clinical Laboratory Science, Faculty of Medical Technology, Teikyo University, Tokyo, Japan, [2] Department of Education Planning and Development, Faculty of Medicine, Toho University, Tokyo, Japan, [3] Department of Internal Medicine, Division of Diabetes, Metabolism and Endocrinology, Toho University, Tokyo, Japan

Graves' hyperthyroidism is caused by autoantibodies directed against the thyroid-stimulating hormone receptor (TSHR) that mimic the action of TSH. The establishment of Graves' hyperthyroidism in experimental animals has proven to be an important approach to dissect the mechanisms of self-tolerance breakdown that lead to the production of thyroid-stimulating TSHR autoantibodies (TSAbs). "Shimojo's model" was the first successful Graves' animal model, wherein immunization with fibroblasts cells expressing TSHR and a major histocompatibility complex (MHC) class II molecule, but not either alone, induced TSAb production in AKR/N (H-2^k) mice. This model highlights the importance of coincident MHC class II expression on TSHR-expressing cells in the development of Graves' hyperthyroidism. These data are also in agreement with the observation that Graves' thyrocytes often aberrantly express MHC class II antigens *via* mechanisms that remain unclear. Our group demonstrated that cytosolic self-genomic DNA fragments derived from sterile injured cells can induce aberrant MHC class II expression and production of multiple inflammatory cytokines and chemokines in thyrocytes *in vitro*, suggesting that severe cell injury may initiate immune responses in a way that is relevant to thyroid autoimmunity mediated by cytosolic DNA signaling. Furthermore, more recent successful Graves' animal models were primarily established by immunizing mice with TSHR-expressing plasmids or adenovirus. In these models, double-stranded DNA vaccine contents presumably exert similar immune-activating effect in cells at inoculation sites and thus might pave the way toward successful Graves' animal models. This review focuses on evidence suggesting that cell injury-derived self-DNA fragments could act as Graves' disease triggers.

Keywords: Graves' disease, thyroid-stimulating hormone receptor, major histocompatibility complex class II, genomic DNA, experimental animal models

OPEN ACCESS

Edited by:
Cesidio Giuliani,
Università degli Studi "G. d'Annunzio" Chieti – Pescara, Italy

Reviewed by:
Eijun Nishihara,
Kuma Hospital, Japan
Takao Ando,
Nagasaki University Hospital, Japan

****Correspondence:***
Koichi Suzuki
koichis0923@med.teikyo-u.ac.jp

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *28 September 2016*
Accepted: *27 October 2016*
Published: *14 November 2016*

Citation:
Luo Y, Yoshihara A, Oda K, Ishido Y and Suzuki K (2016) Excessive Cytosolic DNA Fragments as a Potential Trigger of Graves' Disease: An Encrypted Message Sent by Animal Models. Front. Endocrinol. 7:144. doi: 10.3389/fendo.2016.00144

INTRODUCTION

Graves' disease is a unique human autoimmune disease that involves stimulating autoantibodies directed toward thyroid-stimulating hormone receptors (TSHRs) on the surface of thyroid epithelial cells. This disease occurs in approximately 3% of females and 0.5% of males in the general population (1). Unlike autoantibodies to thyroglobulin (Tg) or thyroid peroxidase (TPO),

thyroid-stimulating TSHR autoantibodies are not just a marker of Graves' disease but are also held directly responsible for the hyperthyroidism that occurs in most of the patients. Moreover, evidence suggests that only TSHR is the primary autoantigen of Graves' disease, whereas immune responses to other thyroid antigens (e.g., Tg and TPO) simply reflect concomitant thyroiditis.

TSHR autoantibody was first discovered in a search for thyroid-stimulating activity in the serum of Graves' disease patients, which was known to stimulate radioiodine release from pre-labeled guinea pig thyroids for a much longer time period than did pituitary TSH treatment (2). This prolonged stimulating activity present in the IgG fraction of Graves' patient serum could compete with TSH for TSHR occupancy, which implies the presence of TSHR antibodies that act as TSHR agonists (3). Thus, in most Graves' disease patients, circulating antibodies that have TSH-like activity continuously stimulate the thyroid. This continuous stimulation results in an enlarged thyroid known as goiter, and these patients have increased iodine uptake and overproduction of thyroid hormone (TH). Typical blood tests for Graves' disease patients show elevated T3 and T4 levels, as well as low TSH (as a result of negative feedback loop) levels (4).

TSHR antibodies (TRAbs) in serum from Graves' disease patients can now be clinically evaluated by non-radioactive third generation assay, in which the autoantibodies inhibit binding of a biotin-labeled human monoclonal thyroid-stimulating antibody M22 to TSHR-coated ELISA plate wells (5). However, TRAbs may or may not initiate a TSH-like intracellular signal. TRAbs that induce a strong TSH-like stimulatory signal are referred to as TSHR-stimulating antibodies/immunoglobulin (TSAbs/TSI), which is the immunological hallmark of Graves' disease. Meanwhile, TRAbs that induce weak or no stimulatory signals are referred to as TSHR-blocking antibodies (TBAbs). The TSAb activity of TRAbs is usually evaluated by their capacity to induce cAMP production in TSHR-expressing cells (6). TSAbs and TBAbs can sometimes coexist in the serum of an individual patient and may change over time. The clinical status of a patient who has both TSAbs and TBAbs presumably depends on the relative concentration and affinity of the predominant antibody type. A shift from TSAbs to TBAbs may occur during spontaneous or treatment-induced remission of Graves' disease that may lead to the subsequent development of hypothyroidism (7). In addition to TRAbs, TPO and/or Tg antibodies are detectable in 25–75% of Graves' disease patients, which is consistent with the lymphocytic infiltration seen in Graves' thyroids and is typically less extensive than that seen for Hashimoto's disease.

Although its characteristic hyperthyroidism symptoms and the availability of sensitive laboratory tests may make the diagnosis of Graves' disease straightforward, the lack of an understanding of the pathogenic mechanisms of this disease has impeded the development of cures. In Graves' disease, immune tolerance toward self-antigen TSHR is obviously dysfunctional, such that, from a classical point of view, endogenous TSHR processed in the cytosol of thyrocytes gives rise to peptides for human leukocyte antigen (HLA) class I presentation to $CD8^+$ T cells. Alternatively, TSHR may be engulfed by antigen-presenting cells (APCs, typically macrophages, dendritic cells, and B cells) where it is digested in the lysosomes and destined for HLA class II presentation to $CD4^+$ T cells. In order to dissect Graves' disease pathogenesis, tremendous efforts have been made to develop experimental Graves' animal models that have indeed provided invaluable insights for understanding the reasons behind the breakdown of self-tolerance.

ANIMAL MODELS OF GRAVES' DISEASE

Autoimmune thyroiditis occurs spontaneously in several animal species (8–10); however, Graves' disease develops spontaneously only in the humans. Conventional animal models of autoimmune thyroiditis that is produced by immunizing animals with Tg or TPO protein have long been available (11). After human TSHR was cloned, similar attempts were made to induce Graves' disease by immunizing animals with human TSHR that was expressed either in a baculovirus expression system or in insect cells (12–16), or purified from cloned human thyroid cells (GEJ) (17). The TSHR is a member of the G protein-coupled receptor superfamily and is coupled with the Gs protein that activates the cAMP-dependent pathway (18). TSHR consists of a short cytoplasmic C-terminal tail, seven transmembrane regions, and a large extracellular horseshoe-shaped leucine-rich repeat region (LRR) known as the ectodomain (**Figure 1**) (18). TSHR reportedly undergoes intramolecular cleavage at some portion of the single-chain polypeptide on the cell surface to form two subunits, such as A and B, which are linked by a hinge of disulfide bonds (19). The extracellular ectodomain (A subunit) of the cleaved receptor is also susceptible to loss by shedding (20, 21) (**Figure 1**). In addition, epitopes for TSAb, but not TBAb, are partially obstructed in wild-type TSHR by the plasma membrane, LRR, or TSHR dimerization. However, the TSAb epitope on the soluble A subunit that is shed from surface TSHR is freely accessible (22). These observations suggest that the shed A subunit, rather than the cell surface full-length TSHR, may be responsible for initiating or amplifying the autoimmune response to the TSHR that in turn leads to Graves' hyperthyroidism. To provide evidence to support this concept, various TSHR ectodomain preparations instead of the full-length TSHR were more frequently used to immunize animals for the development of Graves' disease animal models (12–16). Although serum antibodies and murine monoclonal antibodies against hTSHR were generated in these immunized animals, antibodies with TSAb activity were absent, despite the use of various TSHR preparations with different adjuvants in a variety of mouse strains. The animals did not display increased serum TH, goiter, or thyrocytes hypertrophy either. Human TSHR was thought not to be an authentic autoantigen and thus was unsuitable for inducing autoantibodies in mice. However, even immunization of mice with purified murine TSHR ectodomain expressed in insect cells with an adjuvant failed to induce hyperthyroidism (23).

Purified TSHR peptides expressed in bacteria or insect cells might lack a functional conformation that is needed to induce TSAb in animal models by conventional immunization approaches. To overcome this obstacle, later models used immunization approaches that involved *in vivo* expression of THSR. In these models, animals are injected with transfected

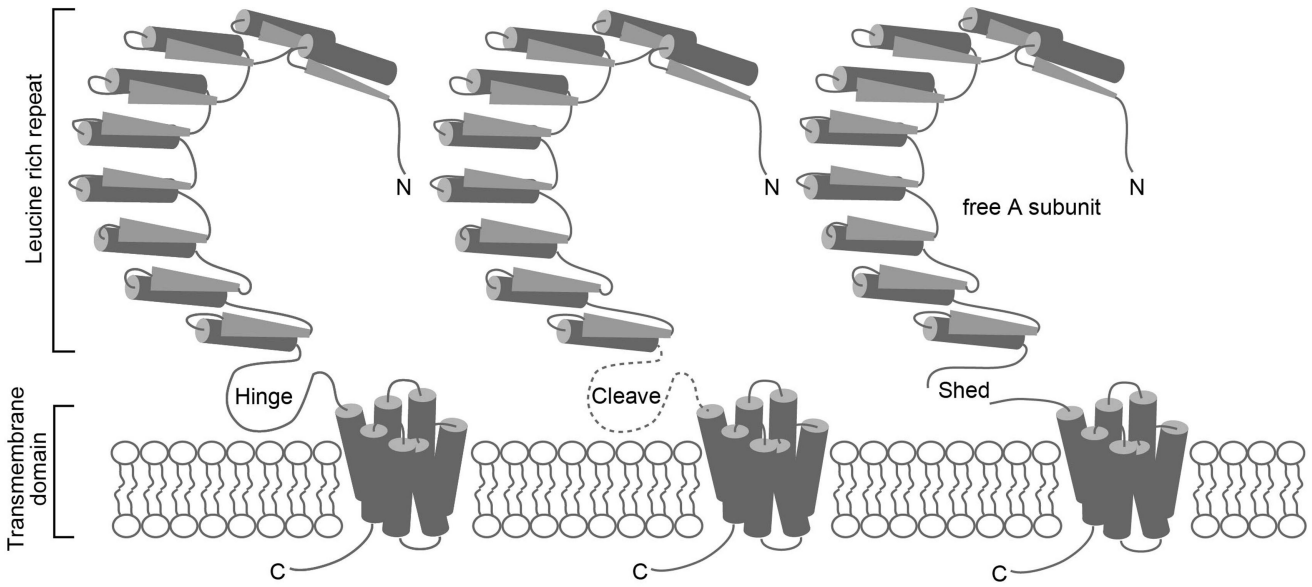


FIGURE 1 | Schematic representations of the TSHR protein, intramolecular cleavage, and A subunit shedding. TSHR consists of an A subunit that has a large extracellular horseshoe-shaped leucine-rich repeat region (LRR) known as the ectodomain, and a B subunit with seven transmembrane regions (left). TSHR undergoes intramolecular cleavage at a hinge-like single-chain polypeptide on the cell surface that connects the A and B subunits (middle). The cleaved receptor is susceptible to loss of the A subunit by shedding (right).

cells stably expressing hTSHR, or with plasmids or adenovirus for transient hTSHR expression. The first authentic animal model of Graves' disease known as "Shimojo's model" was generated by intraperitoneal immunization of female AKR/N (H-2^k) mice with murine fibroblasts that stably expressed full-length hTSHR and a major histocompatibility complex (MHC) class II molecule (24). The use of fibroblasts that express both TSHR and also MHC class II molecules was based on the observation of aberrant expression of MHC class II molecules on thyrocytes from patients with autoimmune thyroid diseases (AITD), including Graves' disease (25). Such observations raised the possibility that TSHR might be presented to immune system by MHC class II-expressing thyrocytes that would break down normal immune tolerance (26). AKR/N (H-2^k) mice were used for these models, as they have a homologous MHC class II I-A molecule that matches the expressed MHC class II molecule on fibroblasts (24). Approximately 20% of immunized mice produced TSAbs and showed increased T4 and T3 levels, as well as goiter with minimal lymphocyte infiltration (24). Intriguingly, immunizing mice with fibroblasts transfected with either TSHR or MHC class II alone did not induce Graves' hyperthyroidism (24), indicating that aberrant expression of MHC class II molecules on cells expressing a native form of the TSHR can induce TSAb production. Shimojo's group later tried the same approach with several different mouse strains that shared the H-2^k haplotype but had different genetic backgrounds and found that, unlike AKR/N mice, C3H/He mice generated TBAbs even in the absence of MHC class II expression (27). However, simultaneous MHC class II expression was needed for TSAb production and development of hyperthyroidism (27). These results suggest that some genetic backgrounds are more susceptible to the induction of TRAbs, while for the development of functional TRAbs, aberrant MHC class II expression is necessary. Since "Shimojo's model" was first described, many other independent groups have tried to reproduce and improve this model (28–31). For example, intraperitoneal immunization with TSHR-expressing M12 (B cells) induced Graves' hyperthyroidism with TSAbs in 100% of immunized BALB/c mice (29, 30). Additionally, intraperitoneal immunization with hTSHR-expressing Chinese hamster ovary (CHO) cells induced Graves' hyperthyroidism in 20% of immunized female Chinese hamsters (28). A noteworthy detail of this study is that the CHO cells used in this immunization approach constitutively expressed MHC class II mRNA as demonstrated by RT-PCR (28). In addition to *in vivo* expression of TSHR, the approaches in these studies shared immunization protocols that included cells that were either stably transfected with MHC class II-encoding cDNA (24, 27, 31, 32) or constitutively expressed MHC class II (28–30). This common feature also leads to a limitation wherein these models are only applicable to syngeneic animals that share the same MHC class II haplotype as the cells used for immunization. Thus, both the success and limitation seem to indicate an important role for aberrant MHC class II expression in the induction of TSAbs and development of Graves' hyperthyroidism.

In order to overcome the strain limitation in Shimojo's approach, novel immunization approaches that relied on intramuscular injection of plasmid vectors encoding TSHR to induce transient TSHR expression by myoblasts at the injection site (33), a method known as "naked" DNA vaccination, were

developed. Intramuscular TSHR DNA vaccination was first tried in female BALB/c mice, which gave rise to TRAbs with TBAb activity in 10 of the 14 immunized mice, whereas weak TSAb activity was detectable in only 1 mouse (34). Severe intrathyroidal lymphocyte infiltration was observed in all the immunized mice, although none developed Graves' hyperthyroidism (34). Thus, these initial attempts using DNA vaccination, despite their ability to generate TRAbs that recognized native TSHR, poorly fulfilled their initial promise of producing TSAbs. The TSHR DNA vaccination approaches were then modified, and different mouse strains were used. In outbred NMRI mice, intramuscular DNA vaccination produced TSAb and hyperthyroidism in 15% of females and 3% of males (35). For BALB/c mice, extensive lymphocyte infiltration was observed in most of the immunized outbred NMRI mice (35). Moreover, intradermal injection of TSHR DNA induced TSAb and hyperthyroidism in inbred female BALB/c mice at an incidence of 27% (36). Indeed, skin could be a better anatomical site for DNA vaccination since the skin is enriched in dendritic cells (Langerhans' cells) that phagocytize and present antigens (36).

Thyroid-stimulating hormone receptor endogenously expressed by vaccination is presumably presented preferentially through the MHC class I antigen pathway; however, the involvement of MHC class II presentation may also be necessary for optimal T cell signaling during TSAb production, as implied in "Shimojo's model." To test this hypothesis, Pichurin et al. constructed a chimeric plasmid that encodes TSHR and the lysosome-associated membrane protein (LAMP)-1, which has a sorting signal that can direct TSHR into lysosomes and, consequently, into the MHC class II presentation pathway. A chimeric TSHR–LAMP-1 plasmid was tested for its efficacy in intramuscular DNA vaccination. Remarkably, TSAb and hyperthyroidism were induced in approximately 20% of female BALA/c mice presumably through hijacking of the TSHR to the MHC class II presentation pathway (37). In contrast, no mice of the same strain injected with wild-type TSHR DNA vaccine showed TSAbs and hyperthyroidism (32). These results indicate that engaging MHC class II presentation facilitates the generation of TSAbs. Additionally, 30% of murine MHC class II knockout HLA-DR3 transgenic NOD mice vaccinated with TSHR DNA showed TSAb induction and Graves' hyperthyroidism (38). These observations, together with the results for BALB/c and outbred mice, indicate that, although theoretically DNA vaccination can be performed in any mouse strain, genetic background remains a decisive factor in DNA vaccination outcomes. Despite the various mouse strains tested and modifications to the immunization protocols, disease incidence induced by DNA vaccination was low (0–30%) (32, 35–42). This low success rate can presumably be attributed to the relatively low TSHR expression efficiency afforded by plasmid vectors.

Substituting adenovirus for plasmid as the vector that encodes THSR cDNA has generally increased the incidence of Graves' hyperthyroidism in mice. In the original report, intramuscular adenovirus (ad)-TSHR immunization induced TSAb and hyperthyroidism symptoms in 55 and 33% of female and male BALB/c mice, respectively. C57BL/6 mice were less susceptible in that only 25% of the females developed hyperthyroidism after the ad-TSHR immunization. Meanwhile, CBA/J, DBA/1J, and SJL/J mice were completely resistant to ad-TSHR-induced hyperthyroidism (43). By adapting the adenovirus vector to express the TSHR A subunit instead of full-length TSHR, the incidence of induced Graves' disease was increased to approximately 65–80% in female BALB/c mice (44, 45). Although intramuscular injection of plasmid-TSHR was less effective than ad-TSHR for inducing Graves' disease in mice, the adoption of intramuscular electroporation for plasmid-TSHR genetic immunization has recently achieved considerable improvement in disease induction as manifested by *in vivo* hTSHR expression and significantly increased disease incidence (46, 47). Surprisingly, TSAbs persisted for more than 8 months after the final electroporation immunization (46), which is in contrast to the transient hyperthyroidism induced by intramuscular immunization wherein TSAb activity often began to decline much earlier or even completely disappeared (48). Another recently reported model of long-term Graves' disease was established by prolonged intramuscular immunization with the ad-TSHR A subunit in female BALB/c mice (49). Long-term Graves' models would be particularly useful for pharmacological analysis and for monitoring treatment response.

ABERRANT EXPRESSION OF MHC CLASS II ON THYROCYTES

By immunizing mice with fibroblasts transfected with both hTSHR and a MHC class II molecule, but not by either alone, Shimojo et al. was the first to successfully generate an authentic Graves' mouse model (24). This model supported a previously proposed hypothesis that epithelial cells from organs that are highly susceptible to organ-specific autoimmunity can be induced to express MHC class II antigens and in turn present antigens to T cells (26). Unlike MHC class I, which is expressed in many types of nucleated cells, MHC class II expression is restricted to professional APCs, such as macrophages, dendritic cells, and B cells. However, aberrant MHC class II expression on thyroid epithelial cells is frequently seen in thyroid autoimmune diseases. Hanafusa et al. used immunofluorescence staining to demonstrate aberrant HLA-DR expression in discrete groups of follicles in 20 of the 26 thyroids from patients with Graves' disease, whereas none of 11 specimens from normal thyroids did (25). Similarly, Jansson et al. reported that HLA-DR-positive thyrocytes were observed in 9 of the 11 specimens of Graves' thyroids by immunohistochemical staining (50). In addition to thyroid autoimmune diseases, aberrant HLA-DR expression on epithelial cells has also been noted in other organ-specific autoimmune diseases, including type I diabetic insulitis (51). Although MHC class II expression is not constitutive, immune mediators can induce MCH class II production in epithelial cells. Interferon (INF)-γ, known as the most prominent MHC class II stimulator, can induce MHC class II on thyrocytes both *in vitro* and *in vivo* (52–54). INF-γ is predominantly produced by lymphocytes as part of innate immune responses (55). Thus, whether aberrant MHC class II expression on Graves' thyrocytes is secondary to coincident lymphocyte infiltration has been wondered. However, so far, the spatial relationship between HLA-DR-positive thyrocytes

and lymphocyte foci in Graves' thyroids remains obscure due to conflicting observations (25, 50).

Besides the undetermined trigger for aberrant MHC class II expression on thyrocytes, little is known about MHC class II antigen presentation in these cells. In contrast to professional APCs, thyrocytes do not have naturally well-adapted machinery for either phagocytosis or antigen processing and presentation, and they do not migrate to lymphoid organs. Nevertheless, there are several lines of evidence to suggest that MHC class II-positive thyrocytes may present peptides to and directly interact with homologous T cells. Induced HLA-DR-positive thyrocytes could promote proliferation of autologous T cells *in vitro*, a phenomenon that does not occur in the absence of HLA-DR expression and is inhibited by HLA-DR monoclonal antibodies (56). Moreover, in a study of 18 Graves' disease patients, expression of HLA-DR antigens on thyrocytes after primary culture in the absence of INFγ was seen in 12 patients, and this expression induced proliferation of autologous T cells derived from both thyroids and peripheral blood (53, 57). T lymphoblast generation was also observed after culturing normal spleen lymphocytes on monolayers of syngeneic thyrocytes for 3 days. Intriguingly, only these T lymphoblasts that had been sensitized on thyrocytes were specifically labeled with fluorescein-conjugated Tg (58). Additionally, primary Graves' thyrocytes were shown to possess phagocytic activity that was enhanced by interleukin-2 and INF-γ and inhibited by antithyroid drugs and steroid medications (59). HLA-DR-positive thyrocytes could present to cloned human T cells an influenza-specific peptide, but not an intact flu virus, and this reaction was blocked by HLA-DR antibodies (60).

Another intriguing question is how aberrantly expressed MHC class II on thyrocytes contributes to breaking self-tolerance. Pichurin et al. demonstrated that hijacking endogenously expressed TSHR into the MHC class II presentation pathway by using a chimeric plasmid encoding both TSHR and the lysosome-directing molecule LAMP was significantly more effective for inducing Graves' hyperthyroidism in BALB/c mice than the use of plasmids encoding wild-type TSHR (37). This finding indicates that the more endogenous antigens entered the MHC class II pathway, the more TSAbs would be generated. Traditionally, immunologists held that MHC class I and II were restricted to the cytosol and endosomes/lysosomes, respectively, for surveying distinct subcellular domains for ligands. However, alternative pathways for delivering exogenous antigens to MHC class I have been characterized and are known as cross-presentation (61). On the other hand, the observation that a large proportion of peptides purified from MHC class II are derived from cytosolic self-proteins (e.g., metabolic enzymes, cytoskeletal proteins, and tumor antigens) indicates that MHC class II may also present endogenous peptides for $CD4^+$ T cell recognition, which has potential relevance to autoimmunity and tumor immunity (62, 63). Endogenously expressed viral proteins were shown to be lysed by MHC class II-restricted virus-specific $CD4^+$ T cells (64), indicating that endogenously expressed proteins can be presented by the MHC class II pathway for $CD4^+$ T cell recognition. Moreover, endogenous antigen presentation by MHC class II could occur through both autophagy-dependent [reviewed in Ref. (65)] and autophagy-independent pathways [reviewed in Ref. (66)]. Meanwhile, the observation that endogenously expressed TSHR A subunits are in general more efficient than non-cleaving TSHR and wild-type TSHR for inducing TSAb and Graves' hyperthyroidism in animal models (45) depicts another possible scenario, in which the shed TSHR A subunit might be internalized by MHC class II-positive thyrocytes and presented through the conventional lysosome/endosome-MHC class II pathway. It is possible that the pathways by which MHC molecules acquire peptides have a significant impact on the generation of peptide diversity that will be ultimately recognized by the T cells and give rise to diverse antibodies.

APC ADAPTATION IN THYROCYTES STIMULATED BY CYTOSOLIC DNA

Major histocompatibility complex class II expression on the transferred fibroblasts was a key factor for the generation of TSAb in "Shimojo's model" (24). In genetic immunization models, vaccines usually consist of TSHR-expressing vectors (plasmids or adenovirus) and sometimes with additional cytokine (such as IL-2, IL-4, and IL-12)-expressing vectors as adjuvants (36). However, none of these vaccines has ever included vectors that express MHC class II. At first glance, MHC class II expression would appear to be irrelevant in the genetic vaccine-induced Graves' animals. Yet in 1999, Suzuki et al. surprisingly found that both MHC class I and II expression was strongly induced on the cell surface of cloned rat thyroid FRTL-5 cells after the cells were transfected with irrelevant or even empty plasmids. In order to dissect the cause for this aberrant MHC expression, they tried different transfection methods and reagents [e.g., lipofection, electroporation, and diethylaminoethyl (DEAE)-dextran] to introduce various DNA substances into FRTL-5 cells. They found that, regardless of the transfection methods and DNA origin, diverse DNA, including bacterial DNA, viral DNA, salmon sperm DNA, calf thymus DNA, self-genomic DNA, plasmid DNA, and artificially synthesized DNA (>25 bp), could induce significant MHC expression on FRTL-5 cells, whereas single-stranded DNA (ssDNA) could not (67). Later studies revealed that a classic double-stranded right-handed helix sense (B-DNA) with a native sugar–phosphate backbone is necessary for the aberrant MHC expression induced by DNA, and the effect was independent of sequence or presence of unmethylated CpG motifs (67–69). Moreover, free DNA in the extracellular medium did not induce MHC expression, indicating that this effect was likely mediated by cytosolic DNA sensors rather than cell surface receptors (67).

Besides MHC molecules, cytosolic DNA induces the expression of an array of molecules in thyrocytes that are involved in antigen-processing and -presenting pathways, such as proteasome protein LMP2, transporter associated with antigen processing (TAP), MHC II-associated invariant chain (Ii), costimulatory molecules (CD80, CD40, CD54, and CD86) (67, 70), and the production of various immune mediators, including type I IFN, TNF-α, and IL-6 (70). These observations indicate that thyrocytes were adapted to behave like APCs with activated innate immune response upon exposure to cytosolic DNA, a phenomenon that

has been widely reproduced in various non-professional APC cells (such as fibroblasts, keratinocytes, epithelial cells, and endothelial cells). Cytosolic DNA similarly enhances APC activity in professional APCs (67–71). Consistent with this finding, T cells were indeed activated to a higher degree, as measured by IL-2 and IFN-γ secretion levels, when they were mixed with peptide-challenged dendritic cells containing cytosolic DNA, compared to those without cytosolic DNA, or those containing ssDNA (71). Based on these findings, it is reasonable to speculate that muscle cells at the injection sites would express MHC class II and undergo adaptations to obtain some APC-like features after immunization with either plasmids or adenovirus (both contain dsDNA structures). These APC-like adaptations occurring in DNA-stimulated cells might have played a significant role to precipitate TSAb generation in animals and may also hold a key to understand the trigger for Graves' disease in the humans.

CELL INJURY INDUCES APC ADAPTATION *VIA* CYTOSOLIC DNA SIGNALS

Thyrocytes would likely encounter DNA in the cytosol following bacteria or virus infection that would introduce foreign DNA (72). Although abundant indirect data suggest the involvement of infecting organisms in the pathogenesis of AITD (72), there is no direct evidence for this possibility and thus the role of infection in AITD remains a subject of debate (73). In addition to foreign DNA, self-DNA that is normally sequestered within the nucleus or in the mitochondria can also enter the cytosol of phagocytes from apoptotic bodies released by dying cells *in vivo*. Phagocytes engulfing these apoptotic bodies from the extracellular medium would eliminate unnecessary DNA through DNase present in the phagolysosomes (74). DNase deficiency leads to the accumulation of large amounts of cytosolic DNA in phagocytes derived from apoptotic cells (75). The ability to remove DNA waste is indispensable for *in vivo* homeostasis. Defective clearance of self-DNA due to mutations in DNase genes is known to be associated with the development of human autoimmune diseases such as systemic lupus erythematosus (SLE) (76, 77), indicating that excessive self-DNA can be a potential trigger for breaking self-tolerance.

Even with normal DNase function, severe cellular injuries, depending on the magnitude, can result in a large amount of DNA waste that outpaces the intrinsic clearance rate of the phagocytes and, thus, would inevitably lead to rapid cytosolic DNA accumulation. In order to demonstrate whether sterile cell injury would cause cytosolic DNA accumulation that is sufficient to induce an APC-like adaptation and stimulate an innate immune response in normal thyrocytes, Kawashima et al. applied electric pulses of increasing intensity to cultured thyrocytes and found that the amount of cytosolic DNA increased in a current intensity-dependent manner and correlated with significantly increased expression of a panel of DNA-inducible molecules, including MHC class II, class II transactivator (CIITA), CD40, CD80, CD86, IFN-β, TNF-α, IL-6, and CCL2 (67, 70). These results support the hypothesis that sterile cell injury can induce aberrant expression of MHC class II and costimulatory molecules and stimulate an innate immune response in the thyrocytes. On the other hand, transfected cytosolic dsDNA, but not ssDNA, suppressed iodide uptake and thyroid-specific functional gene sodium/iodide symporter (*Slc5a5*) expression in the thyrocytes (70). This result is in agreement with previous observations that thyroid function was suppressed when immune activation was

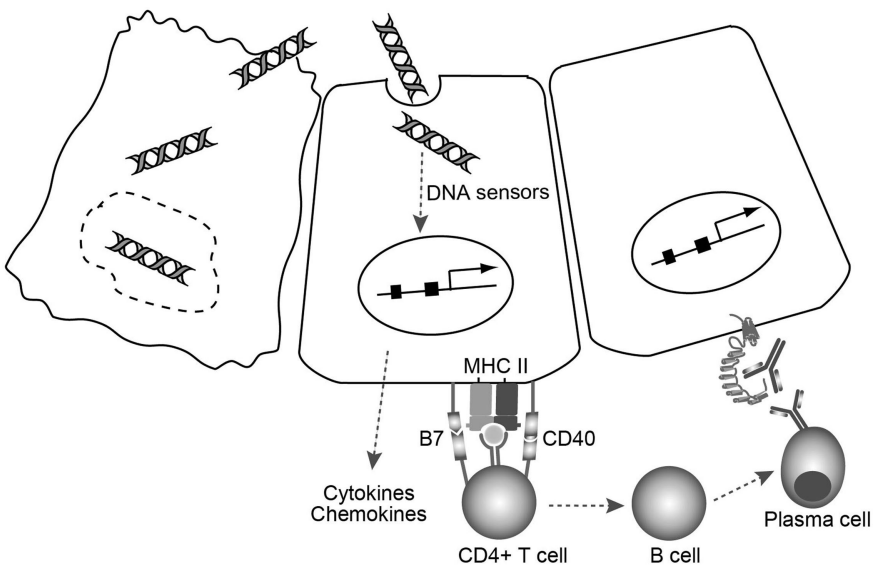


FIGURE 2 | A model for TSAb generation triggered by cell damage-derived self-genomic DNA. Self-genomic DNA released from damaged thyrocytes (left) enters neighboring cells (middle) and induces aberrant expression of MHC class II as well as costimulatory molecules needed for the MHC class II antigen presentation pathway in thyrocytes. At the same time, cytosolic DNA stimulates thyrocytes to produce various proinflammatory cytokines and chemokines that can recruit and activate helper T cells. $CD4^+$ T cells that bind to MHC class II-antigen molecule cause B cell activation. The activated B cells then differentiate into plasma cells that may produce functional TRAbs to stimulate TSHR on the thyrocytes (right).

induced in the thyroid (78). Furthermore, mass spectrometry analysis identified histone H2B as a thyrocyte cytosol protein that bound to a dsDNA Sepharose column (70). Knockdown of histone H2B by siRNA abolished cell injury-induced innate immune activation and increased sodium/iodide symporter (NIS) expression (70), indicating that histone H2B may serve as a cytosolic DNA sensor that mediates the immune-activating effect of DNA in the cytosol.

Based on these studies, a novel model in which cell injury triggers thyroid autoimmune reactions *via* cytosolic DNA signals in the thyrocytes has been proposed (79). In this model (**Figure 2**), fragments of self-genomic DNA released from damaged cells may enter neighboring cells to induce expression of essential molecules in the MHC class II antigen presentation pathway, and the production of type I IFN, proinflammatory cytokines, and chemokines that can recruit and activate lymphocytes and thyrocytes. Endogenous cytosolic TSHR or internalized shed TSHR A subunit may be presented by aberrantly expressed MHC class II on the thyrocytes, with the help of costimulatory molecules to fully activate $CD4^+$ cells. $CD4^+$ T cells that bind to MHC class II-antigen molecules cause activation of B cells, which then differentiate into plasma cells that may produce functional TRAbs to stimulate TSHR. Thus, the cooperation of innate immune activation, inflammation, and aberrant expression of MHC class II and costimulatory molecules will consequently precipitate the generation of TSAbs under an autoimmune-prone genetic background (79).

CONCLUSION

A number of successful mouse/hamster models of Graves' disease have been established during the past two decades. Although each model has some limitations, together they have provided invaluable insight for understanding human Graves' disease. To summarize findings from these models: (1) similar immunization approaches yielded different outcomes in different mouse strains, indicating that genetic background plays an essential role in the development of TSAb; (2) free TSHR A subunits show significant advantages over full-length TSHR for inducing TSAbs, suggesting that the epitopes recognized for the generation of functional TRAbs are likely exposed in the free TSHR A subunit; and (3) Shimojo's model has particularly emphasized that aberrant expression of MHC class II on non-APCs is a contributing factor to Graves' disease. In other words, the involvement of the MHC class II antigen presentation pathway in non-APCs (thyrocytes) may be an important step that leads to breaking of self-tolerance (36, 38, 80).

However, all existing animal models, strictly speaking, are not authentic Graves' models as these animals were artificially immunized with antigens to induce antibody responses. The desired models that reflect the actual pathogenesis of Graves' disease would have spontaneous disease onset without any artificial immunization of the causative antigen, i.e., TSHR, solely by modulating other factors that are suspected to trigger and/or accelerate autoimmune reactions. Such modulations may include the use of thyroid MHC class II/HLA-DR3 or CIITA transgenic mice, transfer of IFN-γ-pretreated syngeneic thyrocytes, increasing intramolecular cleavage and shedding rate of *in vivo* TSHR on the thyrocytes, biasing the immune balance of the extracellular milieu by cytokine/chemokine administration, raising animals with special diets or housing environment, or these conditions in combination. If such a spontaneous model was to be successfully established, even with very low disease incidence will be the true model for understanding Graves' disease pathogenesis and even to prevent and cure Graves' disease.

Inspired by the discovery that cytosolic DNA structures can induce aberrant MHC class II expression on various non-APCs, including thyrocytes, and stimulate the production of various immune mediators, a DNA effect may pave the way for the success of genetic vaccination approaches. The demonstration that sterile cell injury results in cytosolic DNA accumulation correlated with aberrant expression of MHC class II and costimulatory molecules, as well as inflammatory cytokines and chemokines in thyrocytes, raised the possibility that cell injury may affect self-tolerance *via* cytosolic DNA signals. From this perspective, cellular DNA is not just a genetic code but also serves to alert adjacent healthy cells to danger by interacting with a series of cellular sensors. Moreover, if the amount of cytosolic DNA that is derived from severe tissue damage and/or its deficient clearance exceeds a certain threshold, maintenance of self-tolerance may be at risk. The discovery that cell injury-derived excess self-DNA is a potential trigger for initiating thyroid autoimmune reactions may also help to generate an authentic Graves' animal model in the future.

AUTHOR CONTRIBUTIONS

All the authors cooperatively conceived, analyzed, wrote, edited, and approved this review.

REFERENCES

1. Burch HB, Cooper DS. Management of Graves disease: a review. *JAMA* (2015) 314:2544–54. doi:10.1001/jama.2015.16535
2. Adams DD. The presence of an abnormal thyroid-stimulating hormone in the serum of some thyrotoxic patients. *J Clin Endocrinol Metab* (1958) 18:699–712. doi:10.1210/jcem-18-7-699
3. Smith BR, Sanders J, Furmaniak J. TSH receptor antibodies. *Thyroid* (2007) 17:923–38. doi:10.1089/thy.2007.0239
4. Brent GA. Clinical practice. Graves' disease. *N Engl J Med* (2008) 358:2594–605. doi:10.1056/NEJMcp0801880
5. Smith BR, Bolton J, Young S, Collyer A, Weeden A, Bradbury J, et al. A new assay for thyrotropin receptor autoantibodies. *Thyroid* (2004) 14:830–5. doi:10.1089/thy.2004.14.830
6. Lytton SD, Kahaly GJ. Bioassays for TSH-receptor autoantibodies: an update. *Autoimmun Rev* (2010) 10:116–22. doi:10.1016/j.autrev.2010.08.018
7. Wood LC, Ingbar SH. Hypothyroidism as a late sequela in patient with Graves' disease treated with antithyroid agents. *J Clin Invest* (1979) 64:1429–36. doi:10.1172/JCI109601
8. Bigazzi PE, Rose NR. Spontaneous autoimmune thyroiditis in animals as a model of human disease. *Prog Allergy* (1975) 19:245–74.
9. Cohen SB, Weetman AP. Characterization of different types of experimental autoimmune thyroiditis in the buffalo strain rat. *Clin Exp Immunol* (1987) 69:25–32.
10. Damotte D, Colomb E, Cailleau C, Brousse N, Charreire J, Carnaud C. Analysis of susceptibility of NOD mice to spontaneous and experimentally induced thyroiditis. *Eur J Immunol* (1997) 27:2854–62. doi:10.1002/eji.1830271117

11. Kong YC. Experimental autoimmune thyroiditis in the mouse. *Curr Protoc Immunol* (2007) Chapter 15:Unit 15.7. doi:10.1002/0471142735.im1507s78
12. Carayanniotis G, Huang GC, Nicholson LB, Scott T, Allain P, McGregor AM, et al. Unaltered thyroid function in mice responding to a highly immunogenic thyrotropin receptor: implications for the establishment of a mouse model for Graves' disease. *Clin Exp Immunol* (1995) 99:294–302. doi:10.1111/j.1365-2249.1995.tb05548.x
13. Costagliola S, Alcalde L, Tonacchera M, Ruf J, Vassart G, Ludgate M. Induction of thyrotropin receptor (TSH-R) autoantibodies and thyroiditis in mice immunised with the recombinant TSH-R. *Biochem Biophys Res Commun* (1994) 199:1027–34. doi:10.1006/bbrc.1994.1332
14. Wagle NM, Dallas JS, Seetharamaiah GS, Fan JL, Desai RK, Memar O, et al. Induction of hyperthyroxinemia in BALB/C but not in several other strains of mice. *Autoimmunity* (1994) 18:103–12. doi:10.3109/08916939409007983
15. Wagle NM, Patibandla SA, Dallas JS, Morris JC, Prabhakar BS. Thyrotropin receptor-specific antibodies in BALB/cJ mice with experimental hyperthyroxinemia show a restricted binding specificity and belong to the immunoglobulin G1 subclass. *Endocrinology* (1995) 136:3461–9. doi:10.1210/endo.136.8.7628382
16. Wang SH, Carayanniotis G, Zhang Y, Gupta M, McGregor AM, Banga JP. Induction of thyroiditis in mice with thyrotropin receptor lacking serologically dominant regions. *Clin Exp Immunol* (1998) 113:119–25. doi:10.1046/j.1365-2249.1998.00627.x
17. Marion S, Braun JM, Ropars A, Kohn LD, Charreire J. Induction of autoimmunity by immunization of mice with human thyrotropin receptor. *Cell Immunol* (1994) 158:329–41. doi:10.1006/cimm.1994.1280
18. Farid NR, Szkudlinski MW. Minireview: structural and functional evolution of the thyrotropin receptor. *Endocrinology* (2004) 145:4048–57. doi:10.1210/en.2004-0437
19. Loosfelt H, Pichon C, Jolivet A, Misrahi M, Caillou B, Jamous M, et al. Two-subunit structure of the human thyrotropin receptor. *Proc Natl Acad Sci U S A* (1992) 89:3765–9. doi:10.1073/pnas.89.9.3765
20. Couet J, de Bernard S, Loosfelt H, Saunier B, Milgrom E, Misrahi M. Cell surface protein disulfide-isomerase is involved in the shedding of human thyrotropin receptor ectodomain. *Biochemistry* (1996) 35:14800–5. doi:10.1021/bi961359w
21. Couet J, Sar S, Jolivet A, Hai MT, Milgrom E, Misrahi M. Shedding of human thyrotropin receptor ectodomain. Involvement of a matrix metalloprotease. *J Biol Chem* (1996) 271:4545–52. doi:10.1074/jbc.271.8.4545
22. Chazenbalk GD, Pichurin P, Chen CR, Latrofa F, Johnstone AP, McLachlan SM, et al. Thyroid-stimulating autoantibodies in Graves disease preferentially recognize the free A subunit, not the thyrotropin holoreceptor. *J Clin Invest* (2002) 110:209–17. doi:10.1172/JCI15745
23. Vlase H, Matsuoka N, Graves PN, Magnusson RP, Davies TF. Folding-dependent binding of thyrotropin (TSH) and TSH receptor autoantibodies to the murine TSH receptor ectodomain. *Endocrinology* (1997) 138:1658–66. doi:10.1210/endo.138.4.5037
24. Shimojo N, Kohno Y, Yamaguchi K, Kikuoka S, Hoshioka A, Niimi H, et al. Induction of Graves-like disease in mice by immunization with fibroblasts transfected with the thyrotropin receptor and a class II molecule. *Proc Natl Acad Sci U S A* (1996) 93:11074–9. doi:10.1073/pnas.93.20.11074
25. Hanafusa T, Pujol-Borrell R, Chiovato L, Russell RC, Doniach D, Bottazzo GF. Aberrant expression of HLA-DR antigen on thyrocytes in Graves' disease: relevance for autoimmunity. *Lancet* (1983) 2:1111–5. doi:10.1016/S0140-6736(83)90628-1
26. Bottazzo GF, Pujol-Borrell R, Hanafusa T, Feldmann M. Role of aberrant HLA-DR expression and antigen presentation in induction of endocrine autoimmunity. *Lancet* (1983) 2:1115–9. doi:10.1016/S0140-6736(83)90629-3
27. Yamaguchi K, Shimojo N, Kikuoka S, Hoshioka A, Hirai A, Tahara K, et al. Genetic control of anti-thyrotropin receptor antibody generation in H-2K mice immunized with thyrotropin receptor-transfected fibroblasts. *J Clin Endocrinol Metab* (1997) 82:4266–9. doi:10.1210/jcem.82.12.4589
28. Ando T, Imaizumi M, Graves P, Unger P, Davies TF. Induction of thyroid-stimulating hormone receptor autoimmunity in hamsters. *Endocrinology* (2003) 144:671–80. doi:10.1210/en.2002-220582
29. Dogan RN, Vasu C, Holterman MJ, Prabhakar BS. Absence of IL-4, and not suppression of the Th2 response, prevents development of experimental autoimmune Graves' disease. *J Immunol* (2003) 170:2195–204. doi:10.4049/jimmunol.170.4.2195
30. Kaithamana S, Fan J, Osuga Y, Liang SG, Prabhakar BS. Induction of experimental autoimmune Graves' disease in BALB/c mice. *J Immunol* (1999) 163:5157–64.
31. Kita M, Ahmad L, Marians RC, Vlase H, Unger P, Graves PN, et al. Regulation and transfer of a murine model of thyrotropin receptor antibody mediated Graves' disease. *Endocrinology* (1999) 140:1392–8. doi:10.1210/endo.140.3.6599
32. Rao PV, Watson PF, Weetman AP, Carayanniotis G, Banga JP. Contrasting activities of thyrotropin receptor antibodies in experimental models of Graves' disease induced by injection of transfected fibroblasts or deoxyribonucleic acid vaccination. *Endocrinology* (2003) 144:260–6. doi:10.1210/en.2002-220688
33. Tang DC, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature* (1992) 356:152–4. doi:10.1038/356152a0
34. Costagliola S, Rodien P, Many MC, Ludgate M, Vassart G. Genetic immunization against the human thyrotropin receptor causes thyroiditis and allows production of monoclonal antibodies recognizing the native receptor. *J Immunol* (1998) 160:1458–65.
35. Costagliola S, Many MC, Denef JF, Pohlenz J, Refetoff S, Vassart G. Genetic immunization of outbred mice with thyrotropin receptor cDNA provides a model of Graves' disease. *J Clin Invest* (2000) 105:803–11. doi:10.1172/JCI7665
36. Barrett K, Liakata E, Rao PV, Watson PF, Weetman AP, Lymberi P, et al. Induction of hyperthyroidism in mice by intradermal immunization with DNA encoding the thyrotropin receptor. *Clin Exp Immunol* (2004) 136:413–22. doi:10.1111/j.1365-2249.2004.02483.x
37. Pichurin PN, Chazenbalk GD, Aliesky H, Pichurina O, Rapoport B, McLachlan SM. "Hijacking" the thyrotropin receptor: a chimeric receptor-lysosome associated membrane protein enhances deoxyribonucleic acid vaccination and induces Graves' hyperthyroidism. *Endocrinology* (2004) 145:5504–14. doi:10.1210/en.2004-0530
38. Flynn JC, Rao PV, Gora M, Alsharabi G, Wei W, Giraldo AA, et al. Graves' hyperthyroidism and thyroiditis in HLA-DRB1*0301 (DR3) transgenic mice after immunization with thyrotropin receptor DNA. *Clin Exp Immunol* (2004) 135:35–40. doi:10.1111/j.1365-2249.2004.02333.x
39. Baker G, Mazziotti G, von Ruhland C, Ludgate M. Reevaluating thyrotropin receptor-induced mouse models of Graves' disease and ophthalmopathy. *Endocrinology* (2005) 146:835–44. doi:10.1210/en.2004-1015
40. Pichurin P, Chen CR, Pichurina O, David C, Rapoport B, McLachlan SM. Thyrotropin receptor-DNA vaccination of transgenic mice expressing HLA-DR3 or HLA-DQ6b. *Thyroid* (2003) 13:911–7. doi:10.1089/105072503322511300
41. Pichurin P, Pichurina O, Chazenbalk GD, Paras C, Chen CR, Rapoport B, et al. Immune deviation away from Th1 in interferon-gamma knockout mice does not enhance TSH receptor antibody production after naked DNA vaccination. *Endocrinology* (2002) 143:1182–9. doi:10.1210/endo.143.4.8745
42. Pichurin P, Yan XM, Farilla L, Guo J, Chazenbalk GD, Rapoport B, et al. Naked TSH receptor DNA vaccination: a TH1 T cell response in which interferon-gamma production, rather than antibody, dominates the immune response in mice. *Endocrinology* (2001) 142:3530–6. doi:10.1210/endo.142.8.8301
43. Nagayama Y, Kita-Furuyama M, Ando T, Nakao K, Mizuguchi H, Hayakawa T, et al. A novel murine model of Graves' hyperthyroidism with intramuscular injection of adenovirus expressing the thyrotropin receptor. *J Immunol* (2002) 168:2789–94. doi:10.4049/jimmunol.168.6.2789
44. Chen CR, Pichurin P, Chazenbalk GD, Aliesky H, Nagayama Y, McLachlan SM, et al. Low-dose immunization with adenovirus expressing the thyroid-stimulating hormone receptor A-subunit deviates the antibody response toward that of autoantibodies in human Graves' disease. *Endocrinology* (2004) 145:228–33. doi:10.1210/en.2003-1134
45. Chen CR, Pichurin P, Nagayama Y, Latrofa F, Rapoport B, McLachlan SM. The thyrotropin receptor autoantigen in Graves disease is the culprit as well as the victim. *J Clin Invest* (2003) 111:1897–904. doi:10.1172/JCI17069
46. Kaneda T, Honda A, Hakozaki A, Fuse T, Muto A, Yoshida T. An improved Graves' disease model established by using in vivo electroporation exhibited

long-term immunity to hyperthyroidism in BALB/c mice. *Endocrinology* (2007) 148:2335–44. doi:10.1210/en.2006-1077
47. Zhao SX, Tsui S, Cheung A, Douglas RS, Smith TJ, Banga JP. Orbital fibrosis in a mouse model of Graves' disease induced by genetic immunization of thyrotropin receptor cDNA. *J Endocrinol* (2011) 210:369–77. doi:10.1530/JOE-11-0162
48. McLachlan SM, Aliesky HA, Chen CR, Rapoport B. Role of self-tolerance and chronic stimulation in the long-term persistence of adenovirus-induced thyrotropin receptor antibodies in wild-type and transgenic mice. *Thyroid* (2012) 22:931–7. doi:10.1089/thy.2012.0008
49. Holthoff HP, Goebel S, Li Z, Fassbender J, Reimann A, Zeibig S, et al. Prolonged TSH receptor A subunit immunization of female mice leads to a long-term model of Graves' disease, tachycardia, and cardiac hypertrophy. *Endocrinology* (2015) 156:1577–89. doi:10.1210/en.2014-1813
50. Jansson R, Karlsson A, Forsum U. Intrathyroidal HLA-DR expression and T lymphocyte phenotypes in Graves' thyrotoxicosis, Hashimoto's thyroiditis and nodular colloid goitre. *Clin Exp Immunol* (1984) 58:264–72.
51. Pujol-Borrell R, Todd I, Londei M, Foulis A, Feldmann M, Bottazzo GF. Inappropriate major histocompatibility complex class II expression by thyroid follicular cells in thyroid autoimmune disease and by pancreatic beta cells in type I diabetes. *Mol Biol Med* (1986) 3:159–65.
52. Kawakami Y, Kuzuya N, Watanabe T, Uchiyama Y, Yamashita K. Induction of experimental thyroiditis in mice by recombinant interferon gamma administration. *Acta Endocrinol (Copenh)* (1990) 122:41–8.
53. Matsunaga M, Eguchi K, Fukuda T, Kurata A, Tezuka H, Shimomura C, et al. Class II major histocompatibility complex antigen expression and cellular interactions in thyroid glands of Graves' disease. *J Clin Endocrinol Metab* (1986) 62:723–8. doi:10.1210/jcem-62-4-723
54. Todd I, Pujol-Borrell R, Hammond LJ, Bottazzo GF, Feldmann M. Interferon-gamma induces HLA-DR expression by thyroid epithelium. *Clin Exp Immunol* (1985) 61:265–73.
55. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. *Adv Immunol* (2007) 96:41–101. doi:10.1016/S0065-2776(07)96002-2
56. Davies TF. Cocultures of human thyroid monolayer cells and autologous T cells: impact of HLA class II antigen expression. *J Clin Endocrinol Metab* (1985) 61:418–22. doi:10.1210/jcem-61-3-418
57. Eguchi K, Otsubo T, Kawabe Y, Ueki Y, Fukuda T, Matsunaga M, et al. The remarkable proliferation of helper T cell subset in response to autologous thyrocytes and intrathyroidal T cells from patients with Graves' disease. *Clin Exp Immunol* (1987) 70:403–10.
58. Remy JJ, Salamero J, Charreire J. Syngeneic sensitization of mouse lymphocytes on monolayers of thyroid epithelial cells. IX. Thyroid epithelial cells are antigen presenting cells. *Mol Biol Med* (1986) 3:167–79.
59. Matsunaga M, Eguchi K, Fukuda T, Tezuka H, Ueki Y, Kawabe Y, et al. The effects of cytokines, antithyroidal drugs and glucocorticoids on phagocytosis by thyroid cells. *Acta Endocrinol (Copenh)* (1988) 119:413–9.
60. Londei M, Lamb JR, Bottazzo GF, Feldmann M. Epithelial cells expressing aberrant MHC class II determinants can present antigen to cloned human T cells. *Nature* (1984) 312:639–41. doi:10.1038/312639a0
61. Bevan MJ. Cross-priming. *Nat Immunol* (2006) 7:363–5. doi:10.1038/ni0406-363
62. Dani A, Chaudhry A, Mukherjee P, Rajagopal D, Bhatia S, George A, et al. The pathway for MHCII-mediated presentation of endogenous proteins involves peptide transport to the endo-lysosomal compartment. *J Cell Sci* (2004) 117:4219–30. doi:10.1242/jcs.01288
63. Lich JD, Elliott JF, Blum JS. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. *J Exp Med* (2000) 191:1513–24. doi:10.1084/jem.191.9.1513
64. Jacobson S, Sekaly RP, Bellini WJ, Johnson CL, McFarland HF, Long EO. Recognition of intracellular measles virus antigens by HLA class II restricted measles virus-specific cytotoxic T lymphocytes. *Ann N Y Acad Sci* (1988) 540:352–3. doi:10.1111/j.1749-6632.1988.tb27096.x
65. Crotzer VL, Blum JS. Autophagy and its role in MHC-mediated antigen presentation. *J Immunol* (2009) 182:3335–41. doi:10.4049/jimmunol.0803458
66. Leung CS. Endogenous antigen presentation of MHC class II epitopes through non-autophagic pathways. *Front Immunol* (2015) 6:464. doi:10.3389/fimmu.2015.00464
67. Suzuki K, Mori A, Ishii KJ, Saito J, Singer DS, Klinman DM, et al. Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc Natl Acad Sci U S A* (1999) 96:2285–90. doi:10.1073/pnas.96.5.2285
68. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, et al. A toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* (2006) 7:40–8. doi:10.1038/ni1282
69. Stetson DB, Medzhitov R. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. *Immunity* (2006) 24:93–103. doi:10.1016/j.immuni.2005.12.003
70. Kawashima A, Tanigawa K, Akama T, Wu H, Sue M, Yoshihara A, et al. Fragments of genomic DNA released by injured cells activate innate immunity and suppress endocrine function in the thyroid. *Endocrinology* (2011) 152:1702–12. doi:10.1210/en.2010-1132
71. Ishii KJ, Suzuki K, Coban C, Takeshita F, Itoh Y, Matoba H, et al. Genomic DNA released by dying cells induces the maturation of APCs. *J Immunol* (2001) 167:2602–7. doi:10.4049/jimmunol.167.5.2602
72. Tomer Y, Davies TF. Infection, thyroid disease, and autoimmunity. *Endocr Rev* (1993) 14:107–20. doi:10.1210/edrv-14-1-107
73. Hansen PS, Wenzel BE, Brix TH, Hegedus L. Yersinia enterocolitica infection does not confer an increased risk of thyroid antibodies: evidence from a Danish twin study. *Clin Exp Immunol* (2006) 146:32–8. doi:10.1111/j.1365-2249.2006.03183.x
74. Evans CJ, Aguilera RJ. DNase II: genes, enzymes and function. *Gene* (2003) 322:1–15. doi:10.1016/j.gene.2003.08.022
75. Okabe Y, Kawane K, Akira S, Taniguchi T, Nagata S. Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. *J Exp Med* (2005) 202:1333–9. doi:10.1084/jem.20051654
76. Napirei M, Karsunky H, Zevnik B, Stephan H, Mannherz HG, Moroy T. Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet* (2000) 25:177–81. doi:10.1038/76032
77. Yasutomo K, Horiuchi T, Kagami S, Tsukamoto H, Hashimura C, Urushihara M, et al. Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet* (2001) 28:313–4. doi:10.1038/91070
78. Yamazaki K, Suzuki K, Yamada E, Yamada T, Takeshita F, Matsumoto M, et al. Suppression of iodide uptake and thyroid hormone synthesis with stimulation of the type I interferon system by double-stranded ribonucleic acid in cultured human thyroid follicles. *Endocrinology* (2007) 148:3226–35. doi:10.1210/en.2006-1638
79. Kawashima A, Tanigawa K, Akama T, Yoshihara A, Ishii N, Suzuki K. Innate immune activation and thyroid autoimmunity. *J Clin Endocrinol Metab* (2011) 96:3661–71. doi:10.1210/jc.2011-1568
80. Kita-Furuyama M, Nagayama Y, Pichurin P, McLachlan SM, Rapoport B, Eguchi K. Dendritic cells infected with adenovirus expressing the thyrotrophin receptor induce Graves' hyperthyroidism in BALB/c mice. *Clin Exp Immunol* (2003) 131:234–40. doi:10.1046/j.1365-2249.2003.02080.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 03 January 2017
doi: 10.3389/fendo.2016.00167

Building the Case for Insulin-Like Growth Factor Receptor-I Involvement in Thyroid-Associated Ophthalmopathy

Terry J. Smith[1,2] and Joseph A. M. J. L. Janssen[3]*

[1] Department of Ophthalmology and Visual Sciences, Division of Metabolism, Endocrinology, and Diabetes, University of Michigan Medical School, Ann Arbor, MI, USA, [2] Department of Internal Medicine, Division of Metabolism, Endocrinology, and Diabetes, University of Michigan Medical School, Ann Arbor, MI, USA, [3] Department of Internal Medicine, Erasmus Medical Center, Division of Endocrinology, Rotterdam, Netherlands

The pathogenesis of orbital Graves' disease (GD), a process known as thyroid-associated ophthalmopathy (TAO), remains incompletely understood. The thyrotropin receptor (TSHR) represents the central autoantigen involved in GD and has been proposed as the thyroid antigen shared with the orbit that could explain the infiltration of immune cells into tissues surrounding the eye. Another cell surface protein, insulin-like growth factor-I receptor (IGF-IR), has recently been proposed as a second antigen that participates in TAO by virtue of its interactions with anti-IGF-IR antibodies generated in GD, its apparent physical and functional complex formation with TSHR, and its necessary involvement in TSHR post-receptor signaling. The proposal that IGF-IR is involved in TAO has provoked substantial debate. Furthermore, several studies from different laboratory groups, each using different experimental models, have yielded conflicting results. In this article, we attempt to summarize the biological characteristics of IGF-IR and TSHR. We also review the evidence supporting and refuting the postulate that IGF-IR is a self-antigen in GD and that it plays a potentially important role in TAO. The putative involvement of IGF-IR in disease pathogenesis carries substantial clinical implications. Specifically, blocking this receptor with monoclonal antibodies can dramatically attenuate the induction by TSH and pathogenic antibodies generated in GD of proinflammatory genes in cultured orbital fibroblasts and fibrocytes. These cell types appear critical to the development of TAO. These observations have led to the conduct of a now-completed multicenter therapeutic trial of a fully human monoclonal anti-IGF-IR blocking antibody in moderate to severe, active TAO.

Keywords: autoimmune, insulin-like growth factor I receptor, thyrotropin receptor, Graves' disease, hybrid receptor, antibodies, autoantibodies

OPEN ACCESS

Edited by:
Rauf Latif,
Icahn School of Medicine at Mount Sinai, USA

Reviewed by:
Miloš Žarković,
University of Belgrade, Serbia
Yuji Nagayama,
Nagasaki University, Japan

****Correspondence:***
Terry J. Smith
terrysmi@med.umich.edu

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: *15 November 2016*
Accepted: *13 December 2016*
Published: *03 January 2017*

Citation:
Smith TJ and Janssen JAMJL (2017) Building the Case for Insulin-Like Growth Factor Receptor-I Involvement in Thyroid-Associated Ophthalmopathy. Front. Endocrinol. 7:167. doi: 10.3389/fendo.2016.00167

INTRODUCTION

The mechanisms underlying Graves' disease (GD) remain incompletely understood (1). Among the open questions is the basis for loss of immunological tolerance to the thyrotropin receptor (TSHR). Factors underpinning the orbital manifestations of GD, a process known as thyroid-associated ophthalmopathy (TAO), are even less well understood. The unambiguous identification of a pathogenic

autoantigen shared by the orbit and thyroid gland remains to be accomplished. TSHR is the most likely candidate by virtue of its established central role in mediating the hyperthyroidism associated with GD. It has been detected, albeit at very low levels, in the healthy orbit and at somewhat higher levels in orbital tissues during TAO (2). Thyroglobulin (Tg) is another antigen suspect because of its previously unexplained presence in the diseased orbit (3). The insulin-like growth factor-I receptor (IGF-IR) has joined the conversation. It appears to be overexpressed in GD in several cellular compartments (4). Insinuation of IGF-IR in TAO has ignited substantial debate among workers in the field of thyroid autoimmunity (5, 6). In this brief review, we attempt to present a balanced assessment of evidence both refuting and supporting the concept that IGF-IR plays an active and important disease-promoting role in TAO. We also review the proposed mechanisms through which the receptor might serve as a molecular conduit for transducing disease-related signaling initiated by IGF-IR itself and by TSHR. It is possible that IGF-IR might be effectively targeted as therapy for TAO.

GENERAL CONCEPTS ABOUT THE IGF-IR

IGF-IR and the insulin receptor (IR) belong to the family of ligand-activated, plasma membrane-bound tyrosine kinase receptors. Both receptors are widely expressed in many tissues (7). They exhibit substantial structural homology. Depending on which regions are compared, they share sequence identities varying from 41 to 84% (8). Nevertheless, they serve distinct physiological functions *in vivo* (9). Because IGF-I and insulin can produce the same biological responses, and given the widespread tissue distribution of IGF-IR and IR, it has been difficult to determine which of these two receptors mediates a particular response (10). Separation of the different physiological functions mediated through these receptors *in vivo* is imposed by several factors, including their tissue distribution (9). While IR is primarily involved in metabolic actions, IGF-IR promotes cell survival, growth, and differentiation (9). However, IGF-I and insulin can interact promiscuously through both receptors, although with substantially different affinities (11).

IGF-IR like IR comprises two extracellular α-subunits, each containing an IGF-I binding site, and two trans-membrane β-subunits where the catalytic determinants for intrinsic tyrosine kinase activity are located (7). IGF-I elicits multiple biological responses through its high-affinity binding to IGF-IR. Transduction of IGF-I-provoked signaling is initiated through activation of the intrinsic tyrosine kinase and autophosphorylation of IGF-IR. This results in the phosphorylation of multiple tyrosine-containing downstream substrates, including the IRS and Shc proteins (12). Differences in interactions with these substrates arise from the divergent structures of β-subunit and kinase domains in IGF-IR and IR. These variations are hypothesized as being partially responsible for IGF-I and insulin specificity (13). Activated ligand-receptor complexes are thought to be internalized into endosomes (14). Specificity of IGF-I and insulin *in vivo* may result from divergence in the levels of the respective receptors in target tissues coordinated with respective ligand concentration and availability (15). Hybrid receptors comprising both IGF-IR and IR may form in cells expressing both proteins (16). These hybrids are formed during the normal posttranslational processing of both receptors (16). Their formation appears to be stochastic and is therefore receptor concentration-dependent (17). They also appear to determine relative responsiveness to IGF-I and insulin. When levels of IGF-IR exceed those of IRs, IR monomers are mainly present as hybrid receptors (17). These hybrids exhibit high affinity for IGF-I and thus shift the bias away from insulin responsiveness. Although the functional role of hybrid receptors remains incompletely understood, studies have demonstrated that they behave more like IGF-IRs than IRs (16). IGF-IR can also heterodimerize with receptors belonging to other families (18). For example, it can form heterodimers with epidermal growth factor (19). Inhibition of one constituent of these hybrids can shift signaling toward its counterpart receptor (18).

By virtue of its catalytic domain, IGF-IR has traditionally been considered a member of the receptor tyrosine kinase family. It appears that receptor autophosphorylation, particularly at tyrosine residues 1131, 1135, and 1136, is critical to initiation of IGF-I-dependent signaling (20, 21). However, this concept of IGF-IR activation appears to be oversimplified (22). A revised model has now been developed to explain how IGF-I or other activating ligands initiate IGF-IR internalization and subsequent degradation through lysosomal or proteasomal pathways (23). Evidence supports β-arrestins, already implicated in the regulation of G protein-coupled receptors (GPCRs), serving as adaptors between the oncoprotein, E3 ubiquitin ligase Mdm2, and IGF-IR (24). Mdm2 was originally described as controlling IGF-IR ubiquitination and in so doing, promoting its degradation by the proteasome system (25). In this manner, β-arrestin-1 acts as a crucial component in IGF-IR ubiquitination and downregulation. On the other hand, recent studies provide evidence that IGF-IR ubiquitination by β-arrestins/Mdm2 is not simply a receptor desensitization system. While down-regulating IGF-IR from the cell surface and inhibiting its "classical" kinase signaling, β-arrestins activate alternative signaling through MAPK (26). The roles played by β-arrestin-1 in IGF-IR resemble its functions in regulating the behavior of GPCRs. Thus the protein suppresses IGF-IR activity while promoting MAPK signaling (22, 27).

GENERAL CONCEPTS ABOUT THE TSHR

It has been more than 40 years since convincing evidence was put forward for a cell surface-displayed TSHR on thyroid epithelial cells (28). The TSHR gene was first cloned by Vassart and colleagues in 1989 (29). The encoding mRNA has been detected subsequently not only in thyroid tissue but also in multiple fatty depots in animals and human beings (30, 31). Its cognate ligand, TSH' is a glycoprotein hormone produced by thyrotrophs located in the anterior pituitary gland. TSHR plays a central role in the regulation of thyroid growth and function (32). More recently, the receptor was co-crystalized with anti-TSHR antibodies and its structure solved (33, 34). TSHR belongs to the family of rhodopsin-like GPCRs which also includes receptors for luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These proteins possess seven plasma membrane-spanning regions within the so called serpentine domain (35). Surface-displayed

TSHR exists as a multimeric structure (36). The extracellular region represents the amino-terminus containing a high-affinity TSH binding site. The unligated extracellular domain interacts as an inverse agonist with the serpentine domain. TSHR is encoded by a single gene and is synthesized as a single peptide chain that undergoes cleavage into "A" and "B" subunits (37). These are then linked by a disulfide bond. Unlike the receptors for LH and FSH, the extracellular TSHR domain undergoes metalloproteinase-dependent cleavage (38). Some debate exists as to whether the cleavage occurs at the same precise site(s) on the protein and whether the resulting C-peptide sequence is invariant. The specific protease responsible for this cleavage has yet to be identified (39). Evidence has been introduced supporting the concept that this cleaved receptor fragment is shed and provokes the generation of thyroid-stimulating IgGs (TSI) (40). Some authors have expressed the view that the cleaved fragments of TSHR are released into thyroid lymphatics draining into lymph nodes where they are processed by antigen-presenting cells through interactions with mannose receptors (38). TSIs are responsible for the hyperthyroidism associated with GD (40). But not all anti-TSHR antibodies are stimulatory. Some block binding of TSH to the receptor (33) while others are viewed as "neutral." The exact mechanisms involved in the activation of TSHR by either TSH or TSIs remain uncertain although the ligand binding epitopes have been localized (33, 34). Interactions between the different classes of anti-TSHR antibodies and the receptor have also been characterized (41). Signaling downstream from TSHR is complex and involves several pathways that cross talk in patterns that determine the ultimate genes targeted for activation (42–44). Similar but non-identical downstream signaling occurs following TSH and TSI binding to TSHR (45).

EXTRA-THYROIDAL TSHR

Detection of TSHR expression peripheral to the thyroid gland has implicated the protein in an expanding array of biological functions. Particular focus on extra-thyroidal TSHR has involved studies examining the pathogenesis of TAO. Feliciello et al. detected TSHR mRNA in orbital tissues from healthy donors and those with GD (2). TSH promotes lipolysis in rodents and human beings (46, 47). With more advanced techniques of detection, TSHR has been identified, albeit at a very low level, in many fatty and non-adipose tissues (48). The receptor has recently been insinuated in the regulation of bone metabolism (49).

EVIDENCE FOR INTERACTIONS BETWEEN IGF-IR AND TSHR

Accumulating evidence supports the general concept that dissimilar receptor proteins can interact by forming complex signaling partnerships. Recently, Girnita et al. suggested that IGF-IR forms functional hybrids with GPCRs (27). These hybrids utilize components of GPCR signaling and can thus activate pathways conventionally used by GPCRs (27). Multimeric molecular structures of these receptor complexes may help explain the functional interplay that appears to occur between IGF-IR and TSHR pathways. A relationship between IGF-I and TSH signaling was first recognized in 1986 by Ingbar and colleagues (50). They demonstrated that IGF-I could either enhance or antagonize the actions of TSH in cultured thyroid epithelial cells. For instance, IGF-I facilitates the actions of TSH on FRTL-5 cell proliferation while attenuating its induction of sodium/iodide symporter, interactions mediated through PI3 kinase (51). A synergy between the two factors was further demonstrated in the induction of 1, 2-diacylglycerol production in rat thyroid epithelium. In thyroid, the mitogenic activity of IGF-I can be potentiated by TSH (52, 53). TSH induces IRS-2 monoubiquitination in cultured thyroid cells, thereby enhancing IGF-I signaling and mitogenic activity. Both TSH and IGF-I enhance the nuclear content of β-catenin and thus promote Wnt-dependent thyroid epithelial proliferation (54). Conditional knock-out of IGF-IR in thyroid tissue results in increased serum TSH levels and lower serum thyroxine concentrations (55). This profile of circulating hormones suggests relative TSHR insensitivity. In contrast, over-expression of IGF-IR in thyroid amplifies the action of TSH and exaggerates its impact on the synthesis of thyroid hormones (56). We hypothesize that a similar potentiating mechanism might apply following TSHR stimulation by circulating TSI. Further studies will be required to determine whether such a mechanism might underlie the results found in some actions of TSI in the pathogenesis of TAO.

It was uncertain how the two pathways might cross talk at the target cellular level until Tsui and colleagues reported that TSHR and IGF-IR appear to interact directly by forming a protein complex (57). Evidence for these TSHR/IGF-IR complexes was found in orbital fibroblasts and thyroid epithelium utilizing several strategies including co-localization studies with confocal microscopy and co-immunoprecipitation assays. Tsui et al. further demonstrated that a monoclonal blocking antibody directed against IGF-IR could attenuate activation of Erk1/2 by IGF-I, rhTSH, and IgG from patients with GD (57). This report unambiguously demonstrated the functional interdependence of TSHR and IGF-IR and strongly suggested that IGF-IR was transactivated by TSHR. It was followed by several papers confirming (58) and in some cases extending (59, 60) these observations. Evidence for bidirectional crosstalk between the two receptors was demonstrated in another study in orbital fibroblasts (60). IGF-I and TSH were shown to act synergistically in that study by inducing HA production in orbital fibroblasts. Another recent paper contained evidence that inhibiting PI3 kinase and mTOR could attenuate HA accumulation upregulation mediated by these receptors (61). Unfortunately, cultures were exposed to the small molecule inhibitors for many days, inviting criticism of the study design used where conclusions were drawn based on findings that may have been non-specific. Another recent report demonstrated dependence on TSHR in TSHR knock-out mice of IGF-IR protein distribution and levels (62).

EVIDENCE FOR INVOLVEMENT OF IGF-IR IN TAO

Whether a specific autoantigen(s) shared by the orbit and thyroid participates in the pathogenesis of TAO remains an open

question. To our knowledge, demonstration of antigen-specific T cells among those lymphocytes infiltrating the orbit has yet to be unambiguously accomplished. One of the earliest investigators to explore the issue of an ectopically expressed thyroid antigen in the orbit was Kriss (3). He and his colleagues detected Tg in the TAO orbit using thyroidolymphography over four decades ago. More recent studies have substantiated this earlier work (63). Anti-Tg antibodies are commonly detected in thyroid autoimmunity including a substantial proportion of those individuals with GD; however, it is unclear how Tg or the antibodies directed against this protein might play an active role in TAO.

The IGF-I pathway was first implicated in TAO by Weightman et al. (64) who detected immunoglobulins in the sera of individuals with TAO that could displace binding of radiolabeled IGF-I from orbital fibroblast monolayers. This important study was the first to question whether antibodies directed against an IGF-I binding site might be present in these patients. Later studies from Pritchard et al. (65, 66) reported similar results and identified the binding site on orbital fibroblasts as IGF-IR. Their studies indicated that GD-IgG and IGF-I recognize a common binding site. These later studies also revealed that circulating IgGs in GD could induce chemokine expression in TAO orbital fibroblasts, indicating that at least some of these antibodies were biologically active. Pritchard et al. mapped the critical signaling downstream from IGF-IR to the FRAP/mTor/$p70^{S6k}$ pathway. They further demonstrated that the induction of IL-16 and RANTES was inhibited by rapamycin and by transfecting cells with a dominant negative IGF-IR (65, 66). The report also provided evidence for IGF-IR over-expression in these cells when compared to the levels of the receptor in orbital fibroblasts from healthy tissue.

ARE STIMULATORY ANTI-IGF-IR ANTIBODIES DISTINCT FROM TSI?

Reports from Pritchard et al. (65, 66) and Smith and Hoa (67) suggested that IgGs circulating in patients with GD can activate orbital fibroblasts have proven to be controversial (5, 6). The debate rests on whether activating antibodies differing from those against TSHR (i.e., TSI) and instead directly targeting IGF-IR are responsible for the upregulation of cytokine expression and hyaluronan production in orbital fibroblasts (65–67). A major barrier to our quest for the definitive answer derives from an inability to distinguish antibodies that activate IGF-IR from those that merely bind the receptor but fail to initiate signaling. Among the strongest evidence that anti-IGF-IR antibodies are generated in GD are the observations of Weightman et al. (64) and Pritchard et al. (65) demonstrating that GD-IgGs displace IGF-I binding to orbital fibroblasts. More recently, TSHR A-subunit plasmid DNA immunization of mice was shown to result in generation of anti-IGF-IR antibodies (68). Those studies were unable to detect any additional effects of co-immunization with TSHR and IGF-1Rα plasmids on the animal phenotype (68). Thus none of these reports provides insight into whether the anti-IGF-IR antibodies, distinct from TSI, can activate the receptor. Some workers in the field attribute activities of GD-Ig to TSIs rather than IgGs targeting IGF-IR; however, subsequent studies by Pritchard et al. may provide some guidance (69). They demonstrated similar cytokine-inducing activity in synovial fibroblasts from patients with rheumatoid arthritis (RA) when challenged by RA-IgG (69). Their findings indicate that disease-specific IgGs apart from TSI are likely driving these inductions.

More recent studies examining whether activating anti-IGF-IR antibodies are generated in GD have yielded disparate results. Experiments conducted in undifferentiated orbital fibroblasts treated with rhTSH or GD-IgG failed to generate increased levels of HA (70). In contrast, once differentiated into adipocytes, these fibroblasts responded to both (71). Varewijck and colleagues (72) have detected activating anti-IGF-IR antibodies in subsets of patients with GD. They monitored the phosphorylation of multiple tyrosine residues of IGF-IR as the primary read-out for assessing IGF-IR activity (72). In contrast, Minich et al. (73) were unable to distinguish between low levels of anti-IGF-IR IgG activity in healthy controls and those with GD. Their assay was limited to detecting phosphorylation of a single adjacent pair of tyrosine residues (Tyr 1165/1166). They quantified the titer of IGF-IR autoantibodies but their assay was incapable of discriminating between activating and non-activating antibodies. Furthermore, their estimates of the lower limits of antibody titers were based on arithmetic arguments rather than on empirical observations. Another potentially confounding limitation of their study was the likely insensitivity of their assay to low-affinity antibodies. Moreover, effects of stimulating antibodies frequently occur within a narrow concentration range (74) and their studies did not investigate the impact of higher and lower antibody titers. In sum, the conclusions drawn by Minich et al. appear to ignore the likely complex relationship between circulating antibody titers and the magnitude of their biological effects.

Krieger et al. reported an induction by GD-IgG of hyaluronan release from orbital fibroblasts despite an absence of IGF-IR autophosphorylation (59). The authors argued that this scenario rules against an activation of IGF-IR occurring during this action of GD-IgG. They concluded that the actions of GD-IgG must, therefore, be initiated by TSHR rather than through direct interactions with IGF-IR. Yet the authors provided apparently contradictory evidence for receptor activation by demonstrating that the specific IGF-IR tyrosine kinase inhibitor, linsitinib, blocks induction by GD-IgG of hyaluronan production. Thus, we interpret their findings as strongly suggesting that the Western blot assay they used for monitoring IGF-IR phosphorylation failed to detect what might have been low-level but physiologically important receptor activation.

Factors potentially underlying these divergent results include the wide array of assays used, differing target cell types, and the culture conditions used. With regard to culture media, lot to lot variability of endogenous IGF-I, IGF-II, and IGFBP concentrations in the animal sera could alter the background read-out activities observed as well as the magnitude of cellular responses. Thus, it remains possible although unproven that two discrete antibodies generated in GD are at play in the pathogenesis of TAO. This theoretical construct involves one antibody directed at TSHR and the other at IGF-IR. Antibody-induced receptor activation might exhibit tissue specificity. Due to their relatively long half-life of greater than 1 week (52), antibody-dependent

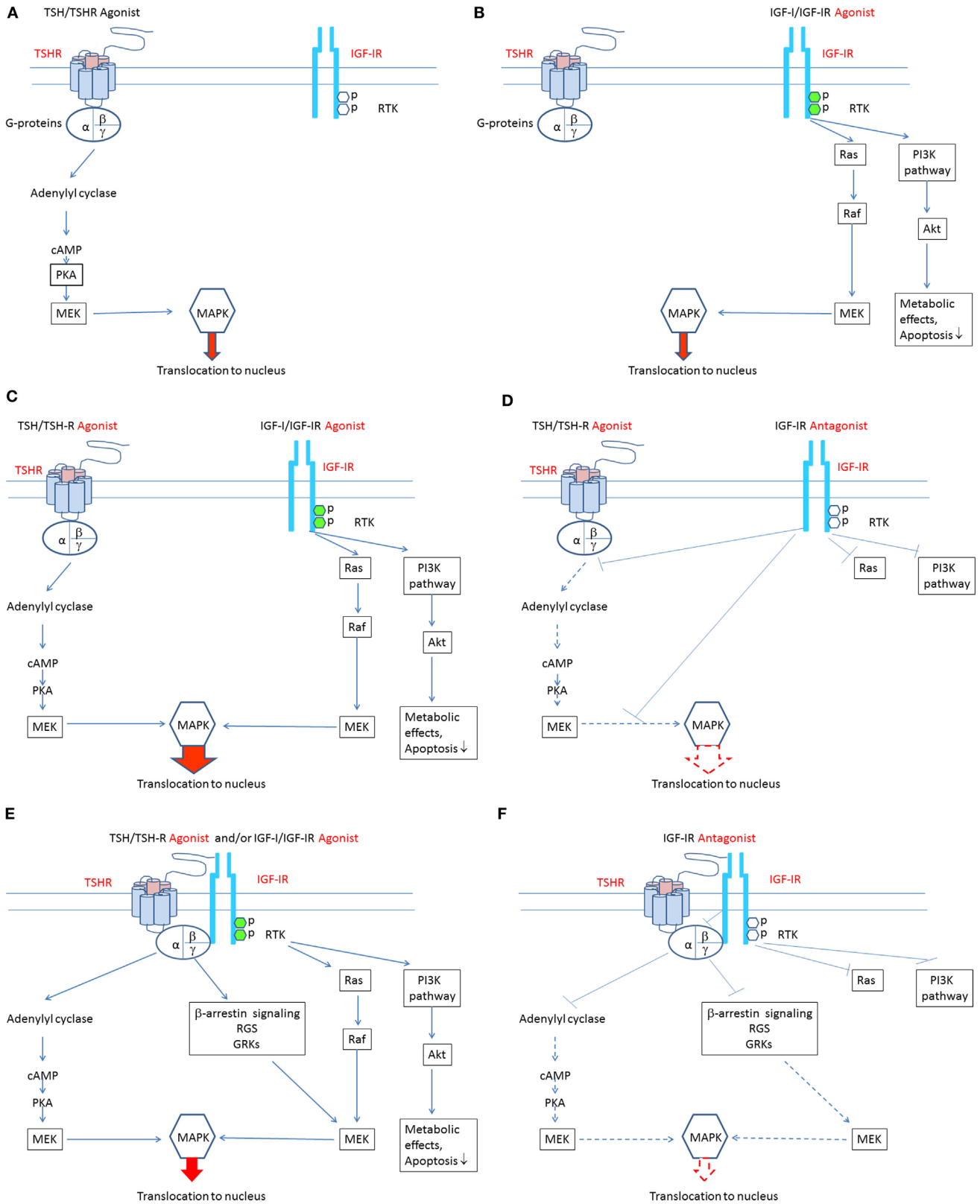


FIGURE 1 | Theoretical mechanisms involved in the crosstalk between insulin-like growth factor-I receptor (IGF-IR) and thyrotropin receptor (TSHR) pathways.

(*Continued*)

FIGURE 1 | **Continued**
(A) Binding of TSH-like agonists to TSHR activates the classical post-receptor pathway by inducing cAMP production, resulting in activation of protein kinase A, mitogen-activated ERK kinase (MEK), and mitogen-activated protein kinase (MAPK). Phosphorylated MAPK translocates to the nucleus where it stimulates several transcription factors regulating gene expression. In this scenario, TSHR activation of its post-receptor pathways is independent of IGF-IR activation.
(B) Binding of IGF-I-like agonists to IGF-IR activates the classical post-receptor pathway by inducing receptor autophosphorylation leading to activation of the phosphoinositide 3-kinase pathway and phosphorylation of Akt. Phosphorylated Akt increases translocation of glucose and is essential for cell survival. Autophosphorylated IGF-IR may also activate Ras which stimulates RAF kinase activity and that of MEK, leading to stimulation of mitogen-activated protein kinase (MAPK). Phosphorylated MAPK translocates to the nucleus where it phosphorylates specific transcription factors regulating gene expression. In this scenario, stimulation of IGF-IR and its post-receptor pathways is independent of TSHR activation. **(C)** Bidirectional crosstalk between the two receptors can occur. IGF-IR agonists can enhance the effects of TSHR agonists. When IGF-IR agonists bind to IGF-IR and TSHR agonists bind to TSHR, additive/synergistic effects can result in higher amplitude stimulation and phosphorylation of MAPK than that resulting from TSHR agonists or IGF-IR agonists acting alone. **(D)** A specific antibody directly targeting IGF-IR might attenuate both IGF-IR- and TSHR-mediated events, thus inhibiting additive/synergistic actions of IGF-IR agonists mediated through TSHR. Blocking IGF-IR with an IGF-IR-specific antagonist may be equivalent to its knockdown. This situation is accompanied by relative TSHR insensitivity (55). **(E)** IGF-IR and TSHR appear to form a physical/functional tyrosine kinase/G protein-coupled receptor (RTK/GPCR) hybrid (57). Such hybrids utilize components of GPCR signaling and can thus activate conventional pathways used by both receptors. Importantly, IGF-IR stimulation by IGF-IR agonists may lead to non-canonical TSHR signaling. Thus, the identical pathways downstream from TSHR may be activated. In this model, signaling downstream from TSHR may occur independently of TSHR activation. Thus, functional IGF-IR/TSHR hybrids may result in bidirectional receptor crosstalk. **(F)** Formation of IGF-IR/TSHR hybrid receptors may underlie inhibitory anti-IGF-IR antibody attenuation of actions initiated at both receptors. Thus, blocking IGF-IR may inhibit both IGF-IR and TSHR-mediated effects. This situation may carry functional equivalence to knocking down IGF-IR, where relative insensitivity to TSH has been demonstrated (55).

activation of TSHR and IGF-IR could be relatively long-lived. It should be stressed that all currently available *in vitro* systems for assessing effects of antibodies on cultured cells may fail to mimic conditions existing *in vivo*. This could result in inaccurate estimates of the events occurring *in situ* within the orbit and potentially in thyroid tissue. In any event, assessment of anti-IGF-IR antibodies activating pathways conventionally used by GPCRs is unprecedented until now.

Most anti-IGF-IR antibodies target the ligand binding site and thus block the binding of endogenous ligands, thereby attenuating receptor activation (52). In contrast, antibodies binding elsewhere on the receptor may be more clinically relevant since they can induce receptor activation (74). Supporting this general concept is the observation that IR-stimulating antibodies activate the receptor by cross-linking subunits rather than by reacting to specific epitopes (74). **Figures 1A–F** summarize putative mechanisms involved in the pathogenesis of TAO. Agonists acting directly at both TSHR and IGF-IR may play roles in stimulating signaling pathways downstream from these receptors. Additional studies will be necessary to untangle what appear to be complex interactions that culminate in the disease.

ULTIMATE TESTING OF THE HYPOTHESIS THAT IGF-IR PARTICIPATES IN TAO

Addressing the question of whether IGF-IR plays an important pathogenic role in TAO and thereby carries potential for therapeutic targeting must await studies conducted *in vivo*. That concept has been tested very recently in a multicenter, placebo controlled, double masked clinical trial of an IGF-IR blocking monoclonal antibody (teprotumumab or RV001) in active, moderate to severe TAO (http://clinicaltrials.gov/show/NCT01868997). The results of that prospective trial should shed new light on this as yet unresolved question.

AUTHOR CONTRIBUTIONS

TS reviewed the literature, drafted portions of the initial manuscript draft, amalgamated the portions of the paper generated by both authors, refined the text, and proof read the final draft. JJ reviewed the literature, drafted portions of the initial manuscript draft, refined the text, generated the theoretical model images, and proof read the final draft.

ACKNOWLEDGMENTS

The authors thank Darla Kroft for her expert help in manuscript preparation.

FUNDING

This work was supported in part by National Institutes of Health grants EY008976 and 5UM1AI110557, Center for Vision core grant EY007002 from the National Eye Institute, an unrestricted grant from Research to Prevent Blindness, and by the Bell Charitable Foundation.

REFERENCES

1. Smith TJ, Hegedus L. Graves' disease. *N Engl J Med* (2016) 375:1552–65. doi:10.1056/NEJMra1510030
2. Feliciello A, Porcellini A, Ciullo I, Bonavolonta G, Avvedimento EV, Fenzi G. Expression of thyrotropin-receptor mRNA in healthy and Graves' disease retro-orbital tissue. *Lancet* (1993) 342:337–8. doi:10.1016/0140-6736(93)91475-2
3. Kriss JP. Radioisotopic thyroidolymphography in patients with Graves' disease. *J Clin Endocrinol Metab* (1970) 31:315–23. doi:10.1210/jcem-31-3-315
4. Smith TJ. Insulin-like growth factor-I regulation of immune function: a potential therapeutic target in autoimmune diseases? *Pharmacol Rev* (2010) 62:199–236. doi:10.1124/pr.109.002469
5. Wiersinga WM. Autoimmunity in Graves' ophthalmopathy: the result of an unfortunate marriage between TSH receptors and IGF-1 receptors? *J Clin Endocrinol Metab* (2011) 96:2386–94. doi:10.1210/jc.2011-0307
6. Smith TJ. Is IGF-I receptor a target for autoantibody generation in Graves' disease? *J Clin Endocrinol Metab* (2013) 98:515–8. doi:10.1210/jc.2013-1004
7. Abbott AM, Bueno R, Pedrini MT, Murray JM, Smith RJ. Insulin-like growth factor I receptor gene structure. *J Biol Chem* (1992) 267:10759–63.

8. Varewijck AJ, Janssen JA. Insulin and its analogues and their affinities for the IGF1 receptor. *Endocr Relat Cancer* (2012) 19:F63–75. doi:10.1530/ERC-12-0026
9. Blakesley VA, Scrimgeour A, Esposito D, Le Roith D. Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cytokine Growth Factor Rev* (1996) 7:153–9. doi:10.1016/1359-6101(96)00015-9
10. Bondy CA, Underwood LE, Clemmons DR, Guler HP, Bach MA, Skarulis M. Clinical uses of insulin-like growth factor I. *Ann Intern Med* (1994) 120:593–601. doi:10.7326/0003-4819-120-7-199404010-00011
11. Froesch ER, Zapf J. Insulin-like growth factors and insulin: comparative aspects. *Diabetologia* (1985) 28:485–93. doi:10.1007/BF00281982
12. Laviola L, Natalicchio A, Giorgino F. The IGF-I signaling pathway. *Curr Pharm Des* (2007) 13:663–9. doi:10.2174/138161207780249146
13. Dupont J, LeRoith D. Insulin and insulin-like growth factor I receptors: similarities and differences in signal transduction. *Horm Res* (2001) 55(Suppl 2):22–6. doi:10.1159/000063469
14. Bevan P. Insulin signalling. *J Cell Sci* (2001) 114:1429–30.
15. Boucher J, Tseng YH, Kahn CR. Insulin and insulin-like growth factor-1 receptors act as ligand-specific amplitude modulators of a common pathway regulating gene transcription. *J Biol Chem* (2010) 285:17235–45. doi:10.1074/jbc.M110.118620
16. LeRoith D, Adamo D, Werner H, Roberts J. Molecular and cellular biolology of the insulin-like growth factors. In: Weintraub BD, editor. *Molecular Endocrinology, Basic Concepts and Clinical Connections*. New York, NY: Raven Press (1995). p. 181–3.
17. Slaaby R, Schaffer L, Lautrup-Larsen I, Andersen AS, Shaw AC, Mathiasen IS, et al. Hybrid receptors formed by insulin receptor (IR) and insulin-like growth factor I receptor (IGF-IR) have low insulin and high IGF-1 affinity irrespective of the IR splice variant. *J Biol Chem* (2006) 281:25869–74. doi:10.1074/jbc.M605189200
18. King ER, Wong KK. Insulin-like growth factor: current concepts and new developments in cancer therapy. *Recent Pat Anticancer Drug Discov* (2012) 7:14–30. doi:10.2174/157489212798357930
19. Morgillo F, Woo JK, Kim ES, Hong WK, Lee HY. Heterodimerization of insulin-like growth factor receptor/epidermal growth factor receptor and induction of survivin expression counteract the antitumor action of erlotinib. *Cancer Res* (2006) 66:10100–11. doi:10.1158/0008-5472.CAN-06-1684
20. Kato H, Faria TN, Stannard B, Roberts CT Jr, LeRoith D. Essential role of tyrosine residues 1131, 1135, and 1136 of the insulin-like growth factor-I (IGF-I) receptor in IGF-I action. *Mol Endocrinol* (1994) 1:40–50. doi:10.1210/mend.8.1.7512194
21. Janssen JA, Hofland LJ, Strasburger CJ, van den Dungen ES, Thevis M. Potency of full- length MGF to induce maximal activation of the IGF-I R is similar to recombinant human IGF-I at high equimolar concentrations. *PLoS One* (2016) 11:e0150453. doi:10.1371/journal.pone.0150453
22. Worrall C, Nedelcu D, Serly J, Suleymanova N, Oprea I, Girnita A, et al. Novel mechanisms of regulation of IGF-1R action: functional and therapeutic implications. *Pediatr Endocrinol Rev* (2013) 10:473–84.
23. Sehat B, Andersson S, Vasilcanu R, Girnita L, Larsson O. Role of ubiquitination in IGF-1 receptor signaling and degradation. *PLoS One* (2007) 2:e340. doi:10.1371/journal.pone.0000340
24. Girnita L, Shenoy SK, Sehat B, Vasilcanu R, Girnita A, Lefkowitz RJ, et al. {beta}-Arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase. *J Biol Chem* (2005) 280:24412–9. doi:10.1074/jbc.M501129200
25. Girnita L, Girnita A, Larsson O. Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor. *Proc Natl Acad Sci USA* (2003) 100:8247–52. doi:10.1073/pnas.1431613100
26. Girnita L, Shenoy SK, Sehat B, Vasilcanu R, Vasilcanu D, Girnita A, et al. Beta-arrestin and Mdm2 mediate IGF-1 receptor-stimulated ERK activation and cell cycle progression. *J Biol Chem* (2007) 282:11329–38. doi:10.1074/jbc.M611526200
27. Girnita L, Worrall C, Takahashi S, Seregard S, Girnita A. Something old, something new and something borrowed: emerging paradigm of insulin-like growth factor type 1 receptor (IGF-1R) signaling regulation. *Cell Mol Life Sci* (2014) 71:2403–27. doi:10.1007/s00018-013-1514-y
28. Amir SM, Carraway TF Jr, Kohn LD, Winand RJ. The binding of thyrotropin to isolated bovine thyroid plasma membranes. *J Biol Chem* (1973) 248:4092–100.
29. Parmentier M, Libert F, Maenhaut C, Lefort A, Gérard C, Perret J, et al. Molecular cloning of the thyrotropin receptor. *Science* (1989) 246:1620–2. doi:10.1126/science.2556796
30. Crisp MS, Lane C, Halliwell M, Wynford-Thomas D, Ludgate M. Thyrotropin receptor transcripts in human adipose tissue. *J Clin Endocrinol Metab* (1997) 82:2003–5.
31. Haraguchi K, Shimura H, Kawaguchi A, Ikeda M, Endo T, Onaya T. Effects of thyrotropin on the proliferation and differentiation of cultured rat preadipocytes. *Thyroid* (1999) 9:613–9. doi:10.1089/thy.1999.9.613
32. Roelfsema F, Veldhuis JD. Thyrotropin secretion patterns in health and disease. *Endocr Rev* (2013) 34:619–57. doi:10.1210/er.2012-1076
33. Sanders P, Young S, Sanders J, Kabelis K, Baker S, Sullivan A, et al. Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. *J Mol Endocrinol* (2011) 46:81–99. doi:10.1530/JME-10-0127
34. Sanders J, Chirgadze DY, Sanders P, Baker S, Sullivan A, Bhardwaja A, et al. Crystal structure of the TSH receptor in complex with a thyroid-stimulating autoantibody. *Thyroid* (2007) 17:395–410. doi:10.1089/thy.2007.0034
35. Cornelis S, Uttenweiler-Joseph S, Panneels V, Vassart G, Costagliola S. Purification and characterization of a soluble bioactive amino-terminal extracellular domain of the human thyrotropin receptor. *Biochemistry* (2001) 40:9860–9. doi:10.1021/bi0107389
36. Urizar E, Montanelli L, Loy T, Bonomi M, Swillens S, Gales C, et al. Glycoprotein hormone receptors: link between receptor homodimerization and negative cooperativity. *EMBO J* (2005) 24:1954–64. doi:10.1038/sj.emboj.7600686
37. Furmaniak J, Hashim FA, Buckland PR, Petersen VB, Beever K, Howells RD, et al. Photoaffinity labelling of the TSH receptor on FRTL5 cells. *FEBS Lett* (1987) 215:316–22. doi:10.1016/0014-5793(87)80169-2
38. Rapoport B, McLachlan SM. TSH receptor cleavage into subunits and shedding of the a-subunit; a molecular and clinical perspective. *Endocr Rev* (2016) 37:114–34. doi:10.1210/er.2015-1098
39. de Bernard S, Misrahi M, Huet JC, Beau I, Desroches A, Loosfelt H, et al. Sequential cleavage and excision of a segment of the thyrotropin receptor ectodomain. *J Biol Chem* (1999) 274:101–7. doi:10.1074/jbc.274.1.101
40. Michalek K, Morshed SA, Latif R, Davies TF. TSH receptor autoantibodies. *Autoimmun Rev* (2009) 9:113–6. doi:10.1016/j.autrev.2009.03.012
41. Miguel RN, Sanders J, Sanders P, Young S, Clark J, Kabelis K, et al. Similarities and differences in interactions of thyroid stimulating and blocking autoantibodies with the TSH receptor. *J Mol Endocrinol* (2012) 49:137–51. doi:10.1530/JME-12-0040
42. Kleinau G, Neumann S, Gruters A, Krude H, Biebermann H. Novel insights on thyroid-stimulating hormone receptor signal transduction. *Endocr Rev* (2013) 34:691–724. doi:10.1210/er.2012-1072
43. Laugwitz KL, Allgeier A, Offermanns S, Spicher K, Van Sande J, Dumont JE, et al. The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. *Proc Natl Acad Sci USA* (1996) 93:116–20. doi:10.1073/pnas.93.1.116
44. Kleinau G, Krause G. Thyrotropin and homologous glycoprotein hormone receptors: structural and functional aspects of extracellular signaling mechanisms. *Endocr Rev* (2009) 30:133–51. doi:10.1210/er.2008-0044
45. Morshed SA, Latif R, Davies TF. Characterization of thyrotropin receptor antibody-induced signaling cascades. *Endocrinology* (2009) 150:519–29. doi:10.1210/en.2008-0878
46. Janson A, Karlsson FA, Micha-Johansson G, Bolme P, Brönnegård M, Marcus C. Effects of stimulatory and inhibitory thyrotropin receptor antibodies on lipolysis in infant adipocytes. *J Clin Endocrinol Metab* (1995) 80:1712–6. doi:10.1210/jcem.80.5.7745024
47. Endo T, Kobayashi T. Expression of functional TSH receptor in white adipose tissues of hyt/hyt mice induces lipolysis in vivo. *Am J Physiol Endocrinol Metab* (2012) 302:E1569–75. doi:10.1152/ajpendo.00572.2011
48. Agretti P, Chiovato L, De Marco G, Marcocci C, Mazzi B, Sellari-Franceschini S, et al. Real-time PCR provides evidence for thyrotropin receptor mRNA expression in orbital as well as in extraorbital tissues. *Eur J Endocrinol* (2002) 147:733–9. doi:10.1530/eje.0.1470733
49. Sun L, Zhu LL, Lu P, Yuen T, Li J, Ma R, et al. Genetic confirmation for a central role for TNFα in the direct action of thyroid stimulating hormone

on the skeleton. *Proc Natl Acad Sci USA* (2013) 110:9891–6. doi:10.1073/pnas.1308336110
50. Tramontano D, Cushing GW, Moses AC, Ingbar SH. Insulin-like growth factor-I stimulates the growth of rat thyroid cells in culture and synergizes the stimulation of DNA synthesis induced by TSH and Graves'-IgG. *Endocrinology* (1986) 119:940–2. doi:10.1210/endo-119-2-940
51. Garcia B, Santisteban P. PI3K is involved in the IGF-I inhibition of TSH-induced sodium/iodide symporter gene expression. *Mol Endocrinol* (2002) 16:342–52. doi:10.1210/mend.16.2.0774
52. Bhaskar V, Goldfine ID, Bedinger DH, Lau A, Kuan HF, Gross LM, et al. A fully human, allosteric monoclonal antibody that activates the insulin receptor and improves glycemic control. *Diabetes* (2012) 61:1263–71. doi:10.2337/db11-1578
53. Fukushima T, Yoshihara H, Furuta H, Kamei H, Hakuno F, Luan J, et al. Nedd4-induced monoubiquitination of IRS-2 enhances IGF signalling and mitogenic activity. *Nat Commun* (2015) 6:6780. doi:10.1038/ncomms7780
54. Sastre-Perona A, Santisteban P. Wnt-independent role of β-catenin in thyroid cell proliferation and differentiation. *Mol Endocrinol* (2014) 28:681–95. doi:10.1210/me.2013-1377
55. Ock S, Ahn J, Lee SH, Kang H, Offermannas S, Ahn HY, et al. IGF-1 receptor deficiency in thyrocytes impairs thyroid hormone secretion and completely inhibits TSH-stimulated goiter. *FASEB J* (2013) 27:4899–908. doi:10.1096/fj.13-231381
56. Clement S, Refetoff S, Robaye B, Dumont JE, Schurmans S. Low TSH requirement and goiter in transgenic mice overexpressing IGF-I and IGF-Ir receptor in the thyroid gland. *Endocrinology* (2001) 142:5131–9. doi:10.1210/endo.142.12.8534
57. Tsui S, Naik V, Hoa N, Hwang CJ, Afifiyan NF, Hikim AS, et al. Evidence for an association between thyroid stimulating hormone and insulin-like growth factor 1 receptors: a tale of two antigens implicated in Graves' disease. *J Immunol* (2008) 181:4397–405. doi:10.4049/jimmunol.181.6.4397
58. Kumar S, Iyer S, Bauer H, Coenen M, Bahn RS. A stimulatory thyrotropin receptor antibody enhances hyaluronic acid synthesis in Graves' orbital fibroblasts: inhibition by an IGF-I receptor blocking antibody. *J Clin Endocrinol Metab* (2012) 97:1681–7. doi:10.1210/jc.2011-2890
59. Krieger CC, Place RF, Bevilacqua C, Marcu-Samuels B, Abel BS, Skarulis MC, et al. TSH/IGF-1 receptor cross talk in Graves' ophthalmopathy pathogenesis. *J Clin Endocrinol Metab* (2016) 101:2340–7. doi:10.1210/jc.2016-1315
60. Krieger CC, Neumann S, Place RF, Marcus-Samuels B, Gershengorn MC. Bidirectional TSH and IGF-1 receptor cross talk mediates stimulation of hyaluronan secretion by Graves' disease immunoglobins. *J Clin Endocrinol Metab* (2014) 100:1071–7. doi:10.1210/jc.2014-3566
61. Zhang L, Grennan-Jones F, Draman MS, Lane C, Morris D, Dayan CM, et al. Possible targets for nonimmunosuppressive therapy of Graves' orbitopathy. *J Clin Endocrinol Metab* (2014) 99:E1183–90. doi:10.1210/jc.2013-4182
62. Atkins SJ, Lentz SI, Fernando R, Smith TJ. Disrupted TSH receptor expression in female mouse lung fibroblasts alters subcellular IGF-1 receptor distribution. *Endocrinology* (2015) 156:4731–40. doi:10.1210/en.2015-1464
63. Marino M, Chiovato L, Lisi S, Altea MA, Marcocci C, Pinchera A. Role of thyroglobulin in the pathogenesis of Graves' ophthalmopathy: the hypothesis of Kriss revisited. *J Endocrinol Invest* (2004) 27:230–6. doi:10.1007/BF03345271
64. Weightman DR, Perros P, Sherif IH, Kendall-Taylor P. Autoantibodies to IGF-1 binding sites in thyroid associated ophthalmopathy. *Autoimmunity* (1993) 16:251–7. doi:10.3109/08916939309014643
65. Pritchard J, Han R, Horst N, Cruikshank WW, Smith TJ. Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the IGF-1 receptor pathway. *J Immunol* (2003) 170:6348–54. doi:10.4049/jimmunol.170.12.6348
66. Pritchard J, Horst N, Cruikshank W, Smith TJ. Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts. *J Immunol* (2002) 168:942–50. doi:10.4049/jimmunol.168.2.942
67. Smith TJ, Hoa N. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, IGF-1 receptor. *J Clin Endocrinol Metab* (2004) 89:5076–80. doi:10.1210/jc.2004-0716
68. Berchner-Pfannschmidt U, Moshkelgosha S, Diaz-Cano S, Edelmann B, Görtz GE, Horstmann M, et al. Comparative assessment of female mouse model of Graves' orbitopathy under different environments, accompanied by proinflammatory cytokine and T-cell responses to thyrotropin hormone receptor antigen. *Endocrinology* (2016) 157:1673–82. doi:10.1210/en.2015-1829
69. Pritchard J, Tsui S, Horst N, Cruikshank WW, Smith TJ. Synovial fibroblasts from patients with rheumatoid arthritis, like fibroblasts from Graves' disease, express high levels of IL-16 when treated with Igs against insulin-like growth factor-1 receptor. *J Immunol* (2004) 173:3564–9. doi:10.4049/jimmunol.173.5.3564
70. van Zeijl CJ, Fliers E, van Koppen CJ, Surovtseva OV, de Gooyer ME, Mourits MP, et al. Effects of thyrotropin and thyrotropin-receptor-stimulating Graves' disease immunoglobulin G on cyclic adenosine monophosphate and hyaluronan production in nondifferentiated orbital fibroblasts of Graves' ophthalmopathy patients. *Thyroid* (2010) 20:535–44. doi:10.1089/thy.2009.0447
71. van Zeijl CJ, Fliers E, van Koppen CJ, Surovtseva OV, de Gooyer ME, Mourits MP, et al. Thyrotropin receptor-stimulating Graves' disease immunoglobulins induce hyaluronan synthesis by differentiated orbital fibroblasts from patients with Graves' ophthalmopathy not only via cyclic adenosine monophosphate signaling pathways. *Thyroid* (2011) 21:169–76. doi:10.1089/thy.2010.0123
72. Varewijck AJ, Boelen A, Lamberts SW, Fliers E, Hofland LJ, Wiersinga WM, et al. Circulating IgGs may modulate IGF-I receptor stimulating activity in a subset of patients with Graves' ophthalmopathy. *J Clin Endocrinol Metab* (2013) 98:769–76. doi:10.1210/jc.2012-2270
73. Minich WB, Dehina N, Welsink T, Schwiebert C, Morgenthaler NG, Köhrle J, et al. Autoantibodies to the IGF1 receptor in Graves' orbitopathy. *J Clin Endocrinol Metab* (2013) 98:752–60. doi:10.1210/jc.2012-1771
74. O'Brien RM, Soos MA, Siddle K. Monoclonal antibodies to the insulin receptor stimulate the intrinsic tyrosine kinase activity by cross-linking receptor molecules. *EMBO J* (1987) 6:4003–10.

Conflict of Interest Statement: TS holds patents related to the detection of antibody-mediated inflammatory autoimmune disorders (US 6936426), the diagnosis and therapy of antibody-mediated inflammatory autoimmune disorders (US 7998681 and US 8153121), and diagnostic methods related to Graves' disease and other autoimmune disorders (US 8178304). No other potential conflict of interest relevant to this article was reported.

frontiers in Endocrinology

REVIEW
published: 28 September 2017
doi: 10.3389/fendo.2017.00254

Autoimmune Thyroid Diseases in Patients Treated with Alemtuzumab for Multiple Sclerosis: An Example of Selective Anti-TSH-Receptor Immune Response

*Mario Rotondi[1], Martina Molteni[1], Paola Leporati[1], Valentina Capelli[2], Michele Marinò[3] and Luca Chiovato[1]**

[1] Unit of Internal Medicine and Endocrinology, Laboratory for Endocrine Disruptors, ICS-Maugeri IRCCS, University of Pavia, Pavia, Italy, [2] Department of Internal Medicine and Therapeutics, and Department of Medical and Surgical Sciences, University of Pavia, Pavia, Italy, [3] Endocrinology Unit I, Department of Clinical and Experimental Medicine, University of Pisa, University Hospital of Pisa, Pisa, Italy

OPEN ACCESS

Edited by:
Cesidio Giuliani,
Università degli Studi "G. d'Annunzio" Chieti – Pescara, Italy

Reviewed by:
Motoyasu Saji,
The Ohio State University Columbus, United States
Amanda Katherine Huber,
University of Michigan, United States

***Correspondence:**
Luca Chiovato
luca.chiovato@icsmaugeri.it

Specialty section:
This article was submitted to Thyroid Endocrinology, a section of the journal Frontiers in Endocrinology

Received: 26 July 2017
Accepted: 15 September 2017
Published: 28 September 2017

Citation:
Rotondi M, Molteni M, Leporati P, Capelli V, Marinò M and Chiovato L (2017) Autoimmune Thyroid Diseases in Patients Treated with Alemtuzumab for Multiple Sclerosis: An Example of Selective Anti-TSH-Receptor Immune Response. Front. Endocrinol. 8:254. doi: 10.3389/fendo.2017.00254

Alemtuzumab, a humanized anti-CD52 monoclonal antibody, is approved for the treatment of active relapsing-remitting multiple sclerosis (MS). Alemtuzumab induces a rapid and prolonged depletion of lymphocytes from the circulation, which results in a profound immuno-suppression status followed by an immune reconstitution phase. Secondary to reconstitution autoimmune diseases represent the most common side effect of Alemtuzumab treatment. Among them, Graves' disease (GD) is the most frequent one with an estimated prevalence ranging from 16.7 to 41.0% of MS patients receiving Alemtuzumab. Thyrotropin (TSH) receptor (R)-reactive B cells are typically observed in GD and eventually present this autoantigen to T-cells, which, in turn, secrete several pro-inflammatory cytokines and chemokines. Given that reconstitution autoimmunity is more frequently characterized by autoantibody-mediated diseases rather than by destructive Th1-mediated disorders, it is not surprising that GD is the most commonly reported side effect of Alemtuzumab treatment in patients with MS. On the other hand, immune reconstitution GD was not observed in a large series of patients with rheumatoid arthritis treated with Alemtuzumab. This negative finding supports the view that patients with MS are intrinsically more at risk for developing Alemtuzumab-related thyroid dysfunctions and in particular of GD. From a clinical point of view, Alemtuzumab-induced GD is characterized by a surprisingly high rate of remission, both spontaneous and after antithyroid drugs, as well as by a spontaneous shift to hypothyroidism, which is supposed to result from a change from stimulating to blocking TSH-receptor antibodies. These immune and clinical peculiarities support the concept that antithyroid drugs should be the first-line treatment in Alemtuzumab-induced Graves' hyperthyroidism.

Keywords: Graves' disease, Alemtuzumab, multiple sclerosis, autoimmune thyroid disease, reconstitution syndrome

ALEMTUZUMAB AS AN IMMUNOMODULATING DRUG

Alemtuzumab is a humanized monoclonal antibody that has been approved for the treatment of active relapsing-remitting (RR) multiple sclerosis (MS) (1, 2). As a main pharmacologic action, Alemtuzumab targets the cell-surface antigen CD52. CD52 is a cell-surface glycoprotein with a still poorly understood function. CD52 is expressed on the surface of more than 95% T and B cells, of monocytes and of some dendritic cells, and, although to a lesser extent, even on natural killer cells and other leukocytes (3). The binding of Alemtuzumab to lymphocytes induces cellular lysis leading to their rapid and prolonged depletion from the circulation (4).

The acute immuno-suppressive effect of Alemtuzumab is followed by the homeostatic reconstitution of immune cells. Typically, monocytes and B cells recover first, followed by CD4+ T cells. Changes in lymphocyte subsets result in an increased number of T regulatory (Treg) cells and of memory T and B lymphocytes; an increased production of anti-inflammatory cytokines also occurs (5). These events produce a profound rebalance of the immune system (6, 7).

Circulating lymphocytes disappear within a few minutes after the administration of Alentuzumab. B cells recover within 3 months and a dominance of mature naïve cells (CD19+ CD23+ CD27−) over the memory B cells occurs. CD4+ T cell counts are restored after 35 months, while CD8+ T cell counts are restored after 20 months. The faster recovery of the latter subset of T cells might be related to the development of autoimmune diseases (8). For at least 9 months after the administration of Alemtuzumab, most circulating T cells are represented by effector memory CD4+ and CD8+ cells. Baker et al. recently described the kinetics of lymphocyte subset reconstitution after Alemtuzumab (9). After depletion, B cells repopulated much more rapidly than T cells in general and Treg in particular (9). In this scenario, the reconstitution of B cells without adequate regulatory control by T cells may explain the high prevalence of post-Alentuzumab autoimmunity (9, 10).

Alentuzumab-induced lymphocytopenia is followed by the homeostatic growth of T cells, which is stimulated by the T cell receptor–self peptide complex. The process results in the appearance of an oligoclonal cell population, which tends to autoreactivity. New T cell populations have typical aspects of memory T cells, such as lower dependency to co-stimulation, need for lower antigen doses than naïve cells, and faster secrection of inflammatory cytokines when re-stimulated (6–8). The above described immune derangements lead to a reduced self-tolerance. In most patients, the proliferation of regulatory lymphocytes is unable to prevent autoimmune deseases, possibly because T cells undergo a faster homeostatic growth, which increases their resistance to regulation (8). Patients who developed autoimmunity after Alentuzumab treatment also show high basal levels of IL-21, a cytokine which increases the growth of auto-reactive T cells. In general, the cytokine expression is skewed to the Th2 profile, in agreement with the high B cell counts (11, 12).

Innate immunity is not affected, and no clinically relevant infection appears after Alentuzumab treatment. This can be due to the maintenance/growth of memory T cells.

The above described immunomodulating actions of Alemtuzumab are responsible for its favorable effects in patients with RRMS (13), but also explain the high prevalence of Alemtuzumab-induced autoimmunity. The latter event received great concern and clinical trials aimed at evaluating potential preventive measures were designed (CAM-THY) (8, 14, 15).

MS AND THYROID DISEASES

Multiple sclerosis is a human chronic inflammatory disease of the central nervous system supposed to be a Th1/Th17 type cell-mediated autoimmune disorder (16, 17). Studies aimed at evaluating whether there is an increased prevalence of autoimmune thyroid diseases (AITDs) in patients with MS as compared with healthy controls reported conflicting results. While early studies found an increased prevalence of AITD in patients with MS, more recent surveys reported rates which are consistent with the AITD prevalence in the general population (18–21). According to more recent views, an increased prevalence of AITD would be observed in family members of patients with MS (22). This is a rather intriguing and yet to be a fully elucidated observation (23, 24).

In addition to studies aimed at evaluating the prevalence of AITD in naive patients with MS, the occurrence of AITD, as a side effect of immunomodulatory treatments for MS, was extensively reported (25–27). At present, we know that treatment with interferon-β (IFN-β) increases the risk for worsening and/or *de novo* appearance of both thyroid autoimmunity and dysfunction. On the other hand, thyroid side effects were not observed following glatiramer acetate (GA) therapy, even in large series of patients with MS who were longitudinally followed for more than 10 years (11, 28, 29).

Specifically designed head-to-head clinical studies demonstrated a similar efficacy of IFN-β and GA, as assessed by their ability to prevent clinical relapses and disease progression in patients with MS (12). However, more effective pharmacologic agents are now available. Among them, Alemtuzumab is currently regarded as an effective second-line treatment in patients with highly active RRMS (1, 2, 13).

TSH-RECEPTOR (TSH-R) AS A MAJOR AUTOANTIGEN IN GRAVES' DISEASE

Graves' disease (GD), also referred to as toxic diffuse goiter, is commonly regarded as an autoimmune organ-specific disease (30–32). The presence of extra-thyroid manifestations, such as Graves' orbitopathy and pretibial myxedema, apparently contradicts this classification, which is, however, justified by the TSH-R being a common antigen shared by the thyroid gland and by extra-thyroid tissues (31). The TSH-R, a G-protein coupled receptor with seven transmembrane-spanning domains and a large extracellular portion, is expressed primarily on the surface of thyroid follicular cells, but it is also present in adipocytes, fibroblast, bone cells, and other sites including the heart (33). TSH-R Antiboides (TRAb) encompass stimulating, blocking, and neutral antibodies. In patiens with GD, TRAb mainly have

thyroid-stimulating activity (TSAb), which results in hyperthyroidism and goiter formation, TSAb bind only the naturally conformed TSH-R and induce cyclic AMP generation, thyroid cell proliferation, and thyroid hormone synthesis and secretion (34, 35). More rarely, and less functionally dominant, TRAb with thyroid blocking activity have been described in patients with GD (36).

Immune system abnormalities in GD are represented by TSH-R-reactive B cells, which escape deletion and eventually present this thyroid autoantigen to T cells. When activated, T cells secrete several pro-inflammatory cytokines and chemokines (37, 38). Hence both B cells and T cells play a central role in perpetuating the autoimmune cascade in GD (11, 37).

AITDs AND ALEMTUZUMAB

Among several autoimmune conditions, which have been reported to occur following Alemtuzumab treatment, the present review will focus on GD, the most frequently observed one. The occurrence of GD in Alemtuzumab-treated patients with secondary progressive MS was first described in 1999 (39). In this early report, a third of MS patients (9/27) receiving the anti-CD52 monoclonal Ab developed GD, with circulating TRAb and hyperthyroidism (39). Besides being the first description, the study by Coles et al. is of great interest, as it clearly shows that GD (and its humoral marker, TRAb) is the most prevalent form of AITD occurring in MS patients treated with Alemtuzumab (39). By contrast, in MS patients, treated with other immunomodulatory therapies (i.e., IFN-β), the most prevalent side effect is represented by euthyroid or hypothyroid autoimmune thyroiditis (26, 27, 40). Thus, a first crucial difference between IFN-β and Alemtuzumab was already evident: they elicited different autoimmune reactions driving the onset of Hashimoto's thyroiditis and GD, respectively.

Subsequent studies, investigating the efficacy and safety of Alemtuzumab therapy in patients with RRMS, confirmed GD as the main autoimmune sequela of this immunomodulatory treatment. In 2008, Coles et al. published a phase 2 clinical trial (CAMMS223) in which 334 patients with RRMS were randomized to receive either IFN-β-1a three times/week or annual cycles of Alemtuzumab (either 12 or 24 mg/day) for 3 years (41). Among other (rare) autoimmune side effects, such as trombocytopenic purpura and glomerulonephritis due to autoantibodies binding the glomerular basal membrane, they reported a significantly higher rate of thyroid autoimmunity in patients treated with Alemtuzumab as opposed to those receiving IFN-β-1a (22.7 versus 2.8%, respectively) (41). In 2012, two phase-3 trials also reported the occurrence of mild to moderate thyroid dysfunction in nearly 18% of RRMS patients treated with Alemtuzumab (1, 2).

The early series of patients included in the CAMMS223 study (2) was further investigated with the specific aim of evaluating thyroid side effects of Alemtuzumab therapy (42). Daniels et al. prolonged the surveillance period of these patients up to a median time of 57.3 months and a maximum of 80.6 months (42). They confirmed that thyroid dysfunctions more frequently occurred in patients treated with Alemtuzumab as compared to those receiving IFN-β-1a. In particular, 34% of patients treated with Alemtuzumab developed thyroid dysfunctions (39% receiving 12 mg and 29% receiving 24 mg) as compared with a 6.5% rate in those treated with IFN-β-1a. As shown in **Table 1**, in the Alemtuzumab treatment group, GD was the most prevalent condition, being experienced by nearly 23% of patients (42). Hypothyroidism was observed in 7.4% patients and destructive thyroiditis with thyrotoxicosis in 4.2% of patients (42).

Some clinical peculiarities of these patients are worth noting. The first episode of thyroid dysfunction was observed starting from the first year after Alemtuzumab administration. Afterward, the episodes' prevalence progressively increased each year for the first 3 years (from 4.6 to 16.1%) with a subsequent decrease in the following 4 years (from 11.3 to 5.9%). There was a higher than expected prevalence (52.7%) of patients in whom Graves' hyperthyroidism (either overt or subclinical) was spontaneously reverted to hypothyroidism (either overt or subclinical) (**Table 2**). An unusual frequency of patients converting from hyperthyroidism to hypothyroidism and *vice versa* was also observed. Importantly, the conversion from hyperthyroidism to hypothyroidism was accompanied by the occurrence of TRAb in nearly 77% of patients (**Table 3**). This observation strongly suggests that the conversion from hyperthyroidism to hypothyroidism was likely due to a shift in the ratio between stimulating and blocking TRAbs. In the routine endocrine practice of sporadic GD, the transition from hyperthyroidism to hypothyroidism is a unusual event, which is mainly observed several years after a successful course of antithyroid drugs and is rarely accompanied by TRAb positivity (43). Taken together, these data indicate that the immune reconstitution occurring after Alemtuzumab treatment is

TABLE 1 | Thyroid dysfunction during Alemtuzumab treatment in patients with multiple sclerosis (MS).

Patients and dysfunctions	(%)
Patients with thyroid dysfunction in the 216 patients cohort	**34**
• Graves' hyperthyroidism	22.4
• Hypothyroidism	7.4
• Destructive thyroiditis	4.2
Type of dysfunction in the 73 affected patients	
• Graves' hyperthyroidism	65.8
• Hypothyroidism	20.5
• Destructive thryoiditis	10.3
• Unknown	1.4
Number of events of thyroid dysfunction between the 102 recorded events	
• Single	70.0
• Multiple	30.0
Type of event among the 102 recorded events	
• Graves' hyperthyroidism	58.8
o Overt	81.7
o Subclinical	18.3
• Hypothyroidism	29.4
• Destructive thryoiditis	9.8
• Unknown	2.0
• Graves' orbitophaty	6.0

Results of the CAMMS223 study as categorized by Daniels et al. (42).

TABLE 2 | Treatment and outcome of Graves' disease developing after Alemtuzumab therapy.

	(%)
Therapy of overt hyperthyroidism in Graves' disease	
• Antithyroid drugs alone	40.1
• Antithyroid drugs + radiometabolic therapy (131-I)	12.2
• Radiometabolic therapy alone (131-I)	6.1
• Surgery (total thyroidectomy)	4.0
Outcome of *overt* hyperthyroidism in Graves' disease	
• Spontaneous resolution with:	36.7
– Euthyroidism	20.4
– Hypothyroidism	16.3
Therapy of *subclinical* hyperthyroidism	
• Antithyroid drugs alone	36.4
Outcome of *subclinical* hyperthyroidism in Graves' disease	63.6
• Spontaneous resolution	18.2
• Subclinical hypothyroidism	18.2
• Overt hypothyroidism	18.2
• Unknown	9.1

Data elaborated from Daniels et al. (42).

TABLE 3 | TSH-receptor antibodies (TRAb) and thyroid dysfunction during Alemtuzumab treatment.

Patients and TRAb	(%)
Positive TRAb at baseline	0.0
De novo positive TRAb	38.0
Thyroid dysfunction and TRAb status	
• Positive TRAb	70.0
Hyperthyroidism and TRAb status	
• Positive TRAb	84.7
Graves' disease and TRAb status	
• Positive TRAb	100
Hypothyroidism and TRAb status	
• Positive TRAb	76.7

Data elaborated from Daniels et al. (42).

TABLE 4 | TPOAb and thyroid dysfunction during Alemtuzumab treatment.

Patients and TPOAb	(%)
Patients with postive TPOAb at baseline	**8.0**
Patients with negative TPOAb at baseline who develop thyroid dysfunction	**31.0**
Patients with postive TPOAb at baseline who develop thyroid dysfunction	**69.0**
• Graves' hyperthyroidism	31.2
• Hypothyroidism	25.0
• Destructive thyroiditis	12.5
• Euthyroidism	31.2
Positive TPOAb at the time of thyroid dysfunction	**69.8**
• Positive TPOAb at baseline	15.1
• *De novo* positive TPOAb	54.8
• Persistently positive TPOAb	30.1
De novo positive TPOAb without thyroid dysfunction	**10.5**

Data elaborated from Daniels et al. (42).

TABLE 5 | Studies reporting Alemtuzumab-related thyroid dysfunction.

Reference	No. of patients	No. of patients who developed thyroid dysfunctions	Thyroid function
Coles et al. (39)	27	9/27 (33%)	Hyperthyroidism: 9 (33%)[a]
Coles et al. (41)	216[a]	49/216 (22.7%)	Hyperthyroidism: 32 (14.8%) Hypothyroidism: 15 (6.9%)
Coles et al. (2)	596	100/596 (16.7%)	Hyperthyroidism: 28 (4.7%) Hypothyroidism: 31 (5.2%)
Cohen et al. (1)	376	68/376 (18%)	Hyperthyroidism: 28 (7%) Hypothyroidism: 18 (5%)
Tuohy et al. (45)	87	35/87 (41%)	Hyperthyroidism: 22 (25.3%) Hypothyroidism: 12 (13.8%)

[a]*One patient developed hypothyroidism before shifting to hyperthyroidism.*

mainly humoral, being directed to the TSH-R as a major autoantigen.

Risk factors for the development of Alemtuzumab-induced GD were a family history of thyroid diseases, female sex, younger age, smoking habit, lower administered dose of the monoclonal Ab, and pretretment postivity for thyroid peroxidase (TPO) antibody (Ab). However, TPOAb had a minor relevance as a risk factor, due to the low frequency of pre-teratment positive results for this autoimmune marker (**Table 4**). Daniels et al. found that only 16/206 (8%) patients were positive for TPOAb at baseline. Among them, the prevalence of thyroid dysfunction after Alentuzumab treatment was 69%, a much higher rate than the 31% one observed in patients who were TPOAb negative at baseline (44). However, the majority (85%) of patients developing a thyroid disorder were negative for TPOAb before Alentuzumab treatment. Therefore, regardless of the pretreatment TPOAb status, patients may develop a thyroid disfunction and should have thyroid function tests performed periodically (41).

The remission rate of Alemtuzumab-induced Graves' hyperthyroidism, either spontaneous or after antithyroid drug treatment (**Table 2**), was also higher (78%) than what commonly observed in the sporadic form of the disease (32).

In a futher observational cohort study, Tuohy et al. (45) re-evaluated 87 patients with RRMS who had been treated with Alemtuzumab in investigator-led studies in Cambridge from 1999 to 2012. This series included 67 patients of the CAMMS224 trial (18) and 20 of the SM3 trial (46). Among the 86 patients who completed the study, 35 (41%) developed a thyroid dysfunction, which was diagnosed as Graves' hyperthyroidism in 22 (63%) and as hypothyroidism with positive tests for TPOAb in 12 (34%) of them. The main limitation of this study is that TRAb were not measured. At the present, large series studies aimed at evaluating the occurrence of thyroid dysfunctions in RRMS patients treated with Alemtuzumab reported a prevalence ranging from 16.7 to 41% (**Table 5**). The remaining published studies on the occurrence of GD in Alemtuzumab-treated patients mainly consist of single case or small series reports, which are summarized in **Table 6**. In the majority of these reports, patients developing AITD were taking Alemtuzumab for a RRMS (47–50). However, reconstitution GD was also described in patients receiving Alemtuzumab for other clinical conditions. The development of Graves' hyperthyroidism was described by

TABLE 6 | Case reports of patients developing autoimmune thyroid diseases following Alemtuzumab administration.

Reference	Case(s)	Disease	Thyroid function	Thyroid Ab	Treatment
Kirk et al. (52)	1 (F)	Kidney transplantation	Hyperthyroidism	TSH-RAb+ TgAb n/a TPOAb n/a	Carbimazole
Aranha et al. (47)	1 (F)	Multiple sclerosis (MS)	Hyperthyroidism	TSH-RAb+ TgAb+ TPOAb+	Carbimazole, thyroidectomy
	2 (F)	MS	Hyperthyroidism	TSH-RAb+ TgAb– TPOAb–	Carbimazole, thyroidectomy
	3 (F)	MS	Hyperthyroidism, then hypothyroidism	TSH-RAb+ TgAb n/a TPOAb n/a	Carbimazole, then levothyroxine
	4 (F)	MS	Hyperthyroidism	TSH-RAb+ TgAb+ TPOAb+	Carbimazole
Tsourdi et al. (48)	1 (M)	MS	Hyperthyroidism	TSH-RAb+ TgAb+ TPOAb+	Thiamazole
	2 (F)	MS	Hyperthyroidism	TSH-RAb+ TgAb+ TPOAb+	Thiamazole, thyroidectomy
	3 (F)	MS	Hyperthyroidism	TSH-RAb+ TgAb+ TPOAb–	Thiamazole, thyroidectomy
	4 (M)	MS	Hyperthyroidism	TSH-RAb+ TgAb– TPOAb+	Thiamazole, thyroidectomy
	5 (F)	MS	Mild hyperthyroidism	TSH-RAb+ TgAb+ TPOAb+	No therapy
Williams et al. (53)	1 (M)	Hematopoietic cell transplantation	Hypothyroidism	TSH-RAb n/a TgAb+ TPOAb+	Levothyroxine
	2 (M)	Hematopoietic cell transplantation	Hyperthyroidism	TSH-RAb– TgAb+ TPOAb+	Methimazole
	3 (M)	Hematopoietic cell transplantation	Hyperthyroidism	TSH-RAb– TgAb+ TPOAb+	Methimazole
Mahzari et al. (49)	1 (M)	MS	Hyperthyroidism, then hypothyroidism	TSH-RAb n/a TgAb n/a TPOAb+	Propylthiouracil, then levothyroxine
	2 (F)	MS	Hyperthyroidism, then hypothyroidism	TSH-RAb n/a TgAb n/a TPOAb+	No therapy, then levothyroxine
	3 (F)	MS	Hyperthyroidism	TSH-RAb n/a TgAb n/a TPOAb+	Methimazole
	4 (F)	MS	Mild hyperthyroidism, then hypothyroidism	TSH-RAb n/a TgAb n/a TPOAb+	No therapy, then levothyroxine
Obermann et al. (50)	1 (M)	MS	Hyperthyroidism	TSH-RAb+ TgAb+ TPOAb+	Carbimazole
	2 (M)	MS	Subclinical hypothyroidism	TSH-RAb– TgAb+ TPOAb+	No therapy

Walsh et al. in 11% of patients treated with Alemtuzumab for vasculitis (51). Other reports include (i) a young kidney transplant recipient who developed GD 4 years after Alemtuzumab treatment (52) and (ii) three pediatric cases of thyroid autoimmune diseases in patients receiving Alemtuzumab after hematopoietic cell transplantation (53).

At variance with all the above described studies, the development of immune reconstition GD was not observed in a large series of patients with rheumatoid arthritis treated with Alemtuzumab (54). These negative findings support the view that patients with MS bear a higher intrinsic risk for the development of Alemtuzumab-related thyroid dysfunctions. Further support to the above statement stems from the notion that while Alemtuzumab is increasingly prescribed in chronic lymphocytic leukemia, no case of Graves' hyperthyroidism has been reported in these patients.

FINAL REMARKS

Reconstitution GD may occur during the recovery phase of Alemtuzumab-induced CD52 cells depletion. Because reconstitution autoimmunity is more frequently related to autoantibody-mediated diseases rather than to destructive, Th1-mediated disorders (i.e., Hashimoto's thyroiditis), it is not surprising that GD is the most commonly reported side effects of Alemtuzumab treatment.

The reason why, as compared with patients bearing other clinical conditions, those with MS carry a higher risk for the development of GD after Alemtuzumab treatment remains unknown. Genetic factors and/or specific clinical aspects of MS, such as the cytokine/chemokine milieu and/or the RR clinical course, might play a role, but there is still no definite proof at this regard.

From a clinical point of view, there are peculiar aspects of Alemtuzumab-induced GD. First, hyperthyroid patients have an unusualy high rate of spontaneous shift to hypothyroidism. This shift is supposed to result from a change from stimulating to blocking TRAb. Second, the remission rate of Graves' hyperthyroidism, both spontaneous and after antithyroid drugs, is unexpectedly high, suggesting a less aggressive disease (14). This observation implies that antithyroid drugs should be the first-line treatment in patients with Alemtuzumab-induced Graves' hyperthyroidism.

AUTHOR CONTRIBUTIONS

LC, MR, MaM, PL, VC, and MiM designed the study and reviewed the literature on thyroid side effects of Alemtuzumab. LC and MR wrote the manuscript. All the authors revised the paper and approved the final edition.

REFERENCES

1. Cohen JA, Coles AJ, Arnold DL, Confavreux C, Fox EJ, Hartung HP, et al. Alemtuzumab versus interferon beta 1a as first-line treatment for patients with relapsing-remitting multiple sclerosis: a randomised controlled phase 3 trial. *Lancet* (2012) 380(9856):1819–28. doi:10.1016/S0140-6736(12)61769-3
2. Coles AJ, Twyman CL, Arnold DL, Cohen JA, Confavreux C, Fox EJ, et al. Alemtuzumab for patients with relapsing multiple sclerosis after disease-modifying therapy: a randomised controlled phase 3 trial. *Lancet* (2012) 380(9856):1829–39. doi:10.1016/S0140-6736(12)61768-1
3. Rao SP, Sancho J, Campos-Rivera J, Boutin PM, Severy PB, Weeden T, et al. Human peripheral blood mononuclear cells exhibit heterogeneous CD52 expression levels and show differential sensitivity to alemtuzumab mediated cytolysis. *PLoS One* (2012) 7(6):e39416. doi:10.1371/journal.pone.0039416
4. Serafini B, Rosicarelli B, Magliozzi R, Stigliano E, Aloisi F. Detection of ectopic B-cell follicles with germinal centers in the meninges of patients with secondary progressive multiple sclerosis. *Brain Pathol* (2004) 14(2):164–74. doi:10.1111/j.1750-3639.2004.tb00049.x
5. Wiendl H, Kieseier B. Multiple sclerosis: reprogramming the immune repertoire with alemtuzumab in MS. *Nat Rev Neurol* (2013) 9(3):125–6. doi:10.1038/nrneurol.2013.2
6. Milo R. Therapeutic strategies targeting B-cells in multiple sclerosis. *Autoimmun Rev* (2016) 15(7):714–8. doi:10.1016/j.autrev.2016.03.006
7. Jones JL, Coles AJ. Mode of action and clinical studies with alemtuzumab. *Exp Neurol* (2014) 262(Pt A):37–43. doi:10.1016/j.expneurol.2014.04.018
8. Weetman A. Immune reconstitution syndrome and the thyroid. *Best Pract Res Clin Endocrinol Metab* (2009) 23(6):693–702. doi:10.1016/j.beem.2009.07.003
9. Baker D, Herrod SS, Alvarez-Gonzalez C, Giovannoni G, Schmierer K. Interpreting lymphocyte reconstitution data from the pivotal phase 3 trials of alemtuzumab. *JAMA Neurol* (2017) 74(8):961–9. doi:10.1001/jamaneurol.2017.0676
10. Steinman L. Induction of new autoimmune diseases after alemtuzumab therapy for multiple sclerosis learning from adversity. *JAMA Neurol* (2017) 74(8):907–8. doi:10.1001/jamaneurol.2017.0325
11. Rotondi M, Stufano F, Lagonigro MS, La Manna L, Zerbini F, Ghilotti S, et al. Interferon-β but not glatiramer acetate stimulates CXCL10 secretion in primary cultures of thyrocytes: a clue for understanding the different risks of thyroid dysfunctions in patients with multiple sclerosis treated with either of the two drugs. *J Neuroimmunol* (2011) 234(1–2):161–4. doi:10.1016/j.jneuroim.2011.01.013
12. Mikol DD, Barkhof F, Chang P, Coyle PK, Jeffery DR, Schwid SR, et al. Comparison of subcutaneous interferon beta-1a with glatiramer acetate in patients with relapsing multiple sclerosis (the REbif vs Glatiramer Acetate in Relapsing MS Disease [REGARD] study): a multicentre, randomised, parallel, open-label trial. *Lancet Neurol* (2008) 7(10):903–14. doi:10.1016/S1474-4422(08)70200-X
13. Coles AJ, Fox E, Vladic A, Gazda SK, Brinar V, Selmaj KW, et al. Alemtuzumab more effective than interferon β-1a at 5-year follow-up of CAMMS223 clinical trial. *Neurology* (2012) 78(14):1069–78. doi:10.1212/WNL.0b013e31824e8ee7
14. Weetman AP. Graves' disease following immune reconstitution or immunomodulatory treatment: should we manage it any differently? *Clin Endocrinol (Oxf)* (2014) 80(5):629–32. doi:10.1111/cen.12427
15. ClinicalTrials.gov. *Keratinocyte Growth Factor to Prevent Autoimmunity After Alemtuzumab Treatment of Multiple Sclerosis*. (2017). NCT01712945. Available from: https://clinicaltrials.gov/ct2/show/NCT01712945
16. Batocchi AP, Rotondi M, Caggiula M, Frisullo G, Odoardi F, Nociti V, et al. Leptin as a marker of multiple sclerosis activity in patients treated with interferon-beta. *J Neuroimmunol* (2003) 139(1–2):150–4. doi:10.1016/S0165-5728(03)00154-1
17. Fletcher JM, Lalor SJ, Sweeney CM, Tubridy N, Mills KH. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol* (2010) 162(1):1–11. doi:10.1111/j.1365-2249.2010.04143.x
18. Durelli L, Oggero A, Verdun E, Isoardo GL, Barbero P, Bergamasco B, et al. Thyroid function and anti-thyroid antibodies in MS patients screened for interferon treatment. A multicenter study. *J Neurol Sci* (2001) 193(1):17–22. doi:10.1016/S0022-510X(01)00637-2
19. Marrie RA, Yu BN, Leung S, Elliott L, Warren S, Wolfson C, et al. The incidence and prevalence of fibromyalgia are higher in multiple sclerosis than the general population: a population-based study. *Mult Scler Relat Disord* (2012) 1(4):162–7. doi:10.1016/j.msard.2012.06.001

20. Niederwieser G, Buchinger W, Bonelli RM, Berghold A, Reisecker F, Költringer P, et al. Prevalence of autoimmune thyroiditis and non-immune thyroid disease in multiple sclerosis. *J Neurol* (2003) 250(6):672–5. doi:10.1007/s00415-003-1053-9
21. Sloka JS, Phillips PW, Stefanelli M, Joyce C. Co-occurrence of autoimmune thyroid disease in a multiple sclerosis cohort. *J Autoimmune Dis* (2005) 2:9. doi:10.1186/1740-2557-2-9
22. Broadley SA, Deans J, Sawcer SJ, Clayton D, Compston DA. Autoimmune disease in first-degree relatives of patients with multiple sclerosis. A UK survey. *Brain* (2000) 123(Pt 6):1102–11. doi:10.1093/brain/123.6.1102
23. Jenkins RC, Weetman AP. Disease associations with autoimmune thyroid disease. *Thyroid* (2002) 12(11):977–88. doi:10.1089/105072502320908312
24. Weetman AP. Diseases associated with thyroid autoimmunity: explanations for the expanding spectrum. *Clin Endocrinol (Oxf)* (2011) 74(4):411–8. doi:10.1111/j.1365-2265.2010.03855.x
25. Caraccio N, Dardano A, Manfredonia F, Manca L, Pasquali L, Iudice A, et al.Long-term follow-up of 106 multiple sclerosis patients undergoing interferon-beta 1a or 1b therapy: predictive factors of thyroid disease development and duration. *J Clin Endocrinol Metab* (2005) 90(7):4133–7. doi:10.1210/jc.2004-2326
26. Monzani F, Caraccio N, Meucci G, Lombardo F, Moscato G, Casolaro A, et al. Effect of 1-year treatment with interferon-beta1b on thyroid function and autoimmunity in patients with multiple sclerosis. *Eur J Endocrinol* (1999) 141(4):325–31. doi:10.1530/eje.0.1410325
27. Rotondi M, Oliviero A, Profice P, Mone CM, Biondi B, Del Buono A, et al. Occurrence of thyroid autoimmunity and dysfunction throughout a nine-month follow-up in patients undergoing interferon-beta therapy for multiple sclerosis. *J Endocrinol Invest* (1998) 21(11):748–52. doi:10.1007/BF03348040
28. Rotondi M, Bergamaschi R, Chiovato L. Disease modifying therapies in multiple sclerosis: could a baseline thyroid check-up drive the therapeutic choice between interferon-β and glatiramer acetate? *Mult Scler* (2014) 20(14):1918–9. doi:10.1177/1352458514533387
29. Frisullo G, Calabrese M, Tortorella C, Paolicelli D, Ragonese P, Annovazzi P, et al. Thyroid autoimmunity and dysfunction in multiple sclerosis patients during long-term treatment with interferon beta or glatiramer acetate: an Italian multicenter study. *Mult Scler* (2014) 20(9):1265–8. doi:10.1177/1352458514521311
30. Leporati P, Groppelli G, Zerbini F, Rotondi M, Chiovato L. Etiopathogenesis of Basedow's disease. Trends and current aspects. *Nuklearmedizin* (2015) 54(5):204–10. doi:10.3413/Nukmed-0739-15-04
31. Marinò M, Latrofa F, Menconi F, Chiovato L, Vitti P. Role of genetic and non-genetic factors in the etiology of Graves' disease. *J Endocrinol Invest* (2015) 38(3):283–94. doi:10.1007/s40618-014-0214-2
32. Bartalena L, Chiovato L, Vitti P. Management of hyperthyroidism due to Graves' disease: frequently asked questions and answers (if any). *J Endocrinol Invest* (2016) 39(10):1105–14. doi:10.1007/s40618-016-0505-x
33. Bahn RS, Dutton CM, Natt N, Joba W, Spitzweg C, Heufelder AE. Thyrotropin receptor expression in Graves' orbital adipose/connective tissues: potential autoantigen in Graves' ophthalmopathy. *J Clin Endocrinol Metab* (1998) 83(3):998–1002. doi:10.1210/jcem.83.3.4676
34. Morshed SA, Davies TF. Graves' disease mechanisms: the role of stimulating, blocking, and cleavage region TSH receptor antibodies. *Horm Metab Res* (2015) 47(10):727–34. doi:10.1055/s-0035-1559633
35. Inaba H, De Groot LJ, Akamizu T. Thyrotropin receptor epitope and human leukocyte antigen in Graves' disease. *Front Endocrinol* (2016) 7:120. doi:10.3389/fendo.2016.00120
36. Chiovato L, Fiore E, Vitti P, Rocchi R, Rago T, Dokic D, et al. Outcome of thyroid function in Graves' patients treated with radioiodine: role of thyroid-stimulating and thyrotropin-blocking antibodies and of radioiodine-induced thyroid damage. *J Clin Endocrinol Metab* (1998) 83(1):40–6. doi:10.1210/jcem.83.1.4492
37. Rotondi M, Chiovato L, Romagnani S, Serio M, Romagnani P. Role of chemokines in endocrine autoimmune diseases. *Endocr Rev* (2007) 28(5):492–520. doi:10.1210/er.2006-0044
38. Rotondi M, Chiovato L. The chemokine system as a therapeutic target in autoimmune thyroid diseases: a focus on the interferon-γ inducible chemokines and their receptor. *Curr Pharm Des* (2011) 17(29):3202–16. doi:10.2174/138161211798157559
39. Coles AJ, Wing M, Smith S, Coraddu F, Greer S, Taylor C, et al. Pulsed monoclonal antibody treatment and autoimmune thyroid disease in multiple sclerosis. *Lancet* (1999) 354(9191):1691–5. doi:10.1016/S0140-6736(99)02429-0
40. Rotondi M, Mazziotti G, Biondi B, Manganella G, Del Buono AD, Montella P, et al. Long-term treatment with interferon-beta therapy for multiple sclerosis and occurrence of Graves' disease. *J Endocrinol Invest* (2000) 23(5):321–4. doi:10.1007/BF03343730
41. Coles AJ, Compston DA, Selmaj KW, Lake SL, Moran S, Margolin DH, et al. Alemtuzumab vs. interferon beta-1a in early multiple sclerosis. *N Engl J Med* (2008) 359(17):1786–801. doi:10.1056/NEJMoa0802670
42. Daniels GH, Vladic A, Brinar V, Zavalishin I, Valente W, Oyuela P, et al. Alemtuzumab-related thyroid dysfunction in a phase 2 trial of patients with relapsing-remitting multiple sclerosis. *J Clin Endocrinol Metab* (2014) 99(1):80–9. doi:10.1210/jc.2013-2201
43. Wood LC, Ingbar SH. Hypothyroidism as a late sequela in patient with Graves' disease treated with antithyroid agents. *J Clin Invest* (1979) 64(5):1429–36. doi:10.1172/JCI109601
44. Topliss DJ. Clinical update in aspects of the management of autoimmune thyroid diseases. *Endocrinol Metab (Seoul)* (2016) 31(4):493–9. doi:10.3803/EnM.2016.31.4.493
45. Tuohy O, Costelloe L, Hill-Cawthorne G, Bjornson I, Harding K, Robertson N, et al. Alemtuzumab treatment of multiple sclerosis: long-term safety and efficacy. *J Neurol Neurosurg Psychiatry* (2015) 86(2):208–15. doi:10.1136/jnnp-2014-307721
46. Somerfield J, Hill-Cawthorne GA, Lin A, Zandi MS, McCarthy C, Jones JL, et al. A novel strategy to reduce the immunogenicity of biological therapies. *J Immunol* (2010) 185(1):763–8. doi:10.4049/jimmunol.1000422
47. Aranha AA, Amer S, Reda ES, Broadley SA, Davoren PM. Autoimmune thyroid disease in the use of alemtuzumab for multiple sclerosis: a review. *Endocr Pract* (2013) 19(5):821–8. doi:10.4158/EP13020.RA
48. Tsourdi E, Gruber M, Rauner M, Blankenburg J, Ziemssen T, Hofbauer LC. Graves' disease after treatment with alemtuzumab for multiple sclerosis. *Hormones (Athens)* (2015) 14(1):148–53. doi:10.14310/horm.2002.1501
49. Mahzari M, Arnaout A, Freedman MS. Alemtuzumab induced thyroid disease in multiple sclerosis: a review and approach to management. *Can J Neurol Sci* (2015) 42(5):284–91. doi:10.1017/cjn.2015.48
50. Obermann M, Ruck T, Pfeuffer S, Baum J, Wiendl H, Meuth SG. Simultaneous early-onset immune thrombocytopenia and autoimmune thyroid disease following alemtuzumab treatment in relapsing-remitting multiple sclerosis. *Mult Scler* (2016) 22(9):1235–41. doi:10.1177/1352458516638558
51. Walsh M, Chaudhry A, Jayne D. Long-term follow-up of relapsing/refractory anti-neutrophil cytoplasm antibody associated vasculitis treated with the lymphocyte depleting antibody alemtuzumab (CAMPATH-1H). *Ann Rheum Dis* (2008) 67(9):1322–7. doi:10.1136/ard.2007.081661
52. Kirk AD, Hale DA, Swanson SJ, Mannon RB. Autoimmune thyroid disease after renal transplantation using depletional induction with alemtuzumab. *Am J Transplant* (2006) 6(5 Pt 1):1084–5. doi:10.1111/j.1600-6143.2006.01258.x
53. Williams KM, Dietzen D, Hassoun AA, Fennoy I, Bhatia M. Autoimmune thyroid disease following alemtuzumab therapy and hematopoietic cell transplantation in pediatric patients with sickle cell disease. *Pediatr Blood Cancer* (2014) 61(12):2307–9. doi:10.1002/pbc.25102
54. Lorenzi AR, Clarke AM, Wooldridge T, Waldmann H, Hale G, Symmons D, et al. Morbidity and mortality in rheumatoid arthritis patients with prolonged therapy-induced lymphopenia: twelve-year outcomes. *Arthritis Rheum* (2008) 58(2):370–5. doi:10.1002/art.23122

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership