HHS Public Access

Author manuscript

Nat Rev Endocrinol. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

Nat Rev Endocrinol. 2015 March ; 11(3): 171–181. doi:10.1038/nrendo.2014.226.

Recognizing the Putative Role for TSH Receptor Expressing Fibrocytes in Thyroid-Associated Ophthalmopathy may solve several mysteries

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Abstract

Thyroid-associated ophthalmopathy (TAO) remains the vexing and undertreated ocular component of Graves' disease where orbital tissues undergo extensive remodeling. We have recently introduced the concept that $CD34^+$ fibrocytes, bone marrow derived monocyte lineage precursor cells express the thyrotropin receptor (TSHR) and several other proteins traditionally thought to be expressed uniquely in the thyroid. TSHR-engaged fibrocytes generate extremely high levels of several inflammatory cytokines. Acting in concert with TSHR, the insulin-like growth factor 1 receptor (IGF-1R) expressed by fibrocytes appears to be necessary for TSHR-dependent cytokine production since anti-IGF-1R blocking antibodies attenuate these actions of TSH. Further, circulating fibrocytes become more abundant and appear to infiltrate orbital connective tissues in TAO where they may transition to $CD34^+$ fibroblasts. We currently postulate that the infiltration of fibrocytes into the orbit and their unique biosynthetic repertoire and proinflammatory/profibrotic phenotype account for the characteristic properties exhibited by orbital connective tissues that render them susceptible to TAO. Further, it may be possible to utilize these very recent insights to therapeutically target pathogenic orbital fibrocytes selectively utilizing recently developed biologic agents which interfere with TSHR and IGF-1R signaling.

Introduction

Thyroid-associated ophthalmopathy (TAO) represents an autoimmune process that affects the orbit and adjacent tissues of the upper face in the syndrome known as Graves' disease (GD)[1,2]. Unlike most other forms of autoimmunity, TAO is associated with a distinctive, frequently predictable, and self-limited pattern of disease activity[3]. Initially, it is manifested by an often-intense active phase where inflammation, tissue expansion, and orbital congestion predominate. This eventually culminates in a chronic, stable period which is typified by the absence of changing symptoms or ocular measurements. This phase may be dominated by mechanical restrictions resulting from frank fibrosis[4]. Uncertainty persists as to why the orbital contents are singled out for involvement in GD. Most investigators have

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The author affirms no competing interests.

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focused on the orbital connective tissue as the primary target of immune reactivity in TAO[2,5] while a minority believes that the extraocular muscles rather than orbital connective tissue are primarily involved[6]. Their contention is based largely on the detection of serum antibodies in individuals with TAO directed against several muscle proteins. Intrinsic differences appear to distinguish orbital fat and connective tissues from those inhabiting other regions of the body. We postulate that the unique properties of orbital fibroblasts may render the orbit susceptible to the characteristic inflammation, volume expansion, and remodeling in TAO[4,7]. A characteristic pattern of inflammatory gene expression can be detected in affected orbital connective tissues including several immediate early genes[8,9]. Many believe that expression by orbital tissue of the thyrotropin receptor (TSHR), the central pathogenic autoantigen in GD, underlies TAO. In this review, I attempt to introduce the concept that $CD34^+$ fibrocytes are potential participants in the pathogenesis of TAO. These monocyte lineage-derived cells express several “thyroid specific” proteins, including TSHR, and have been identified in the diseased orbit[10]. Because of their striking immunological and biosynthetic properties, we believe that a strong case can be made for fibrocytes as prime candidates for therapeutic targeting in TAO.

Early studies associating TSHR with TAO

Cloning of TSHR by Parmentier and colleagues[11] represented a pivotal breakthrough in gaining insight into normal thyroid glandular function and the pathogenesis of GD. This notable accomplishment opened the flood gates through which molecular interrogation of the genetics, protein structure/function, patterns of TSHR expression and signaling could be accomplished. Detailed characterization of TSHR structure has allowed a better understanding of the molecular rationale for its involvement in thyroid autoimmunity[12]. It has yielded detailed mapping of immunogenic determinants of the receptor protein and has disclosed details of how TSHR signals within the thyroid epithelial cell[13–19]. These insights have proven invaluable to better defining the molecular basis for hyperthyroidism in GD. In that process, activating antibodies, known as thyroid-stimulating immunoglobulins (TSI) underlie excessive production of thyroid hormones by mimicking the actions of TSH[20]. But unlike the well-regulated production of TSH in the anterior pituitary, TSI production and activity are not subjected to negative feedback. Thus the trophic actions of TSI can result in the hyper-metabolism characteristic of GD[1]. Of potential importance, TSHR signaling in thyroid epithelial cells differs when TSH or TSIs activate the receptor[19].

Substantial but largely circumstantial evidence supports involvement of TSI and TSHR in TAO. Recent detection of TSHR outside the anatomic boundaries of the thyroid and insinuation of its functions in extra-thyroidal tissues has generated interest in critically examining broader physiological and pathological roles for the protein. Finding TSHR in orbital fat and extra-ocular muscles[21] has prompted inquiry into whether it might be involved in TAO. Theoretically, the receptor could participate in at least two pathogenically distinct ways: first as an autoantigen shared by the thyroid and orbit and second, as a molecular conduit for conveying disease-related molecular signaling. With regard to the former function, this concept is born out of its central involvement in the hyperthyroidism associated with GD[1]. Several studies have demonstrated detectable TSI in the vast majority of individuals with TAO[22,23]. However, evidence that TSI levels or activity correlate

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longitudinally within individuals with regard to disease duration, severity, or activity has not been conclusive. Further, TAO appearing in individuals where TSI is undetectable, in those with euthyroid GD, or in Hashimoto's thyroiditis complicates any model insinuating TSHR in orbital disease pathogenesis. Direct involvement of TSHR in TAO is implied by the promiscuous expression of TSHR in orbital connective tissues. Feliciello *et al*[21] reported detecting TSHR mRNA in orbital connective tissue shortly after the molecular cloning of TSHR. That report contained evidence for orbital expression of TSHR mRNA regardless of the health status of the donor. Derivative orbital fibroblasts also express the receptor at extremely low levels *in vitro* compared to those found on thyroid epithelium[24]. This expression can be fractionally enhanced by subjecting the fibroblast cultures to conditions favoring their differentiation into adipocytes. But TSHR expression has also been identified in several other tissues and cell-types and thus appears to not be anatomically restricted to tissues ordinarily associated with the manifestations of GD[25–29]. Reports have appeared suggesting direct effects of TSH and TSI on cultured orbital fibroblasts,[30–33]. Both provoke the synthesis of cytokines and hyaluronan (HA), the principal glycosaminoglycan accumulating in TAO[4,30,33,34]. Whether signaling downstream from TSHR in extra-thyroidal tissues diverges when TSH or TSI activate the receptor, as appears to be the case in thyroid[19], has not yet been determined. Potentially important differences in post-TSHR signaling in thyroid epithelium versus non-thyroid cells have recently been identified. TSH induces gene expression in some cells that do not express adenylate cyclase and where TSHR signaling is thus completely independent of cAMP generation[30]. Definition of how the receptor might play a role in TAO is currently the topic of substantial debate that centers not only on examining TSHR within the orbit but also in other extra-thyroidal compartments, such as circulating cells originating in the bone marrow. An expanded appreciation for the potential roles assumed by TSHR in extra-thyroidal tissues should better define the potential for targeting this receptor as a therapeutic strategy in TAO.

Chemoattractants and their potential role in T cells trafficking to the orbit

Another aspect of localized tissue activation in TAO relates to the underlying mechanisms through which professional immune cells such as T and B cells, monocytes, and mast cells might be trafficked to the orbit. An array of chemoattractants expressed by orbital fibroblasts, endothelial cells, and vascular smooth muscle could help explain the infiltration of $CD4^+$ and $CD8^+$T cells that have been identified in affected tissues[35–38]. These include molecules that are classified as chemokines by virtue of their harboring signature protein sequences and others that lack these motifs but function similarly. Sciaky *et al*[39] reported that orbital fibroblasts activated by IL-1β express IL-16, a non-chemokine chemoattractant that specifically targets $CD4^+$ T cells, and regulated on activation, normal T cell expressed and secreted (RANTES or CCL5), a member of the IL-8 super-family that targets T cells and monocytes. Moreover, these activated fibroblasts exhibit substantially enhanced T cell migration *in vitro*. Activation of orbital fibroblasts through the CD40/CD40 ligand bridge results in the elaboration of IL-6, IL-8, and macrophage chemoattractant protein-1 (MCP-1)[40]. On the other hand, interferon γ in combination with TNF-α induces both CCL2 and CXCL10 production in these cells[41]. Thus under the influence of several stimuli, orbital fibroblasts can express and release multiple chemokines and other chemoattractants that may

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underlie the orbital tissue infiltration with a variety of immune mononuclear cells in TAO. What remains uncertain is the means by which balance among multiple signals is achieved in these infiltrating target cells.

Emergence of insulin-like growth factor 1 receptor (IGF-1R) as a putative autoantigen in TAO

A long-standing and as yet unanswered question concerns whether the pathogenic TSI might initiate or in some manner promote mononuclear cell infiltration of the orbit. Another concerns the possibility that additional autoantigens besides TSHR could share relevance to GD and TAO. Among these, Weightman *et al*[42] demonstrated that IgGs from patients with TAO could displace radiolabeled IGF-1 from the surfaces of cultured orbital fibroblasts. This finding implied that a specific IGF-1 binding site might be recognized by these antibodies. While their studies failed to identify or characterize the nature of this fibroblast site, they introduced the then novel concept that the IGF-1 pathway might be involved in TAO. We now know that multiple proteins bind IGF-1 and IGF-2 with high affinity and high specificity, including the IGF-1 receptor (IGF-1R) and the family of IGF-binding proteins[43]. Subsequent studies by Pritchard *et al*[44] revealed that IgGs from patients with GD but not those from healthy individuals could induce IL-16 and RANTES in TAO orbital fibroblasts by activating the Akt/FRAP/mTor/p70^{s6k} pathway. The responses elicited by IgGs were inhibited by rapamycin and glucocorticoids. In later studies, these activating IgGs were found to target IGF-1R[45]. Moreover, this receptor was found to be over-expressed by TAO fibroblasts compared to those from healthy controls. Induction of neither chemoattractant could be detected in fibroblasts from healthy donors.

Finding that antibodies harbored by individuals with GD and TAO can target IGF-1R is congruent with the initial studies[42]. Subsequent reports have either confirmed that anti-IGF-1R antibodies can be detected differentially in at least a subset of patients with GD[46] or have failed to distinguish the presence of anti-IGF-1R antibodies in healthy individuals compared to those with the disease[47]. A number of technical issues may have confounded attempts to replicate the original findings of Weightman *et al*[42] and Pritchard *et al*[44,45]. It would appear that further refinement and standardization of experimental conditions and designing assays that are best suited for detecting these antibodies will be necessary before any firm conclusions can be drawn about whether IGF-1R represents a second antigen in GD and TAO[48]. Of particular importance would be a means of distinguishing anti-IGF-1R antibodies that can activate the receptor from those that merely bind but fail to initiate signaling. In any event, identification of the potential involvement of IGF-1R and its signaling pathway in TAO and in mediating actions initiated by TSH/TSI on extra-thyroidal tissues has led to the conduct of the largest therapeutic trial for TAO ever conducted in North America [http://clinicaltrials.gov/show/NCT01868997]. This ongoing, multicenter placebo controlled study examines the safety and efficacy of the human monoclonal anti-IGF-1R blocking antibody, teprotumamab, in severe, active TAO.

Besides the evidence that IGF-1R and antibodies directed against it are involved in the immune pathogenesis of GD, the IGF-1 pathway appears to share an intimate relationship with the physiological regulation by TSH of thyroid function. Initial evidence for this

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involvement was provided by Tramontano *et a*[49] who demonstrated either synergy or antagonism between TSH action and IGF-1 on thyroid epithelial cells in culture. They demonstrated that IGF-1 could enhance the effects of TSH on FRTL-5 cell proliferation. In contrast, IGF-1 dampens the induction by TSH of NIS, an action mediated by PI_3 kinase[50]. IGF-1 and TSH synergistically enhance 1,2-diacylglycerol production in rat thyroid cells[51]. Very recently, Sastre-Perona and Santisteban[52] reported that TSH and IGF-1 increase the nuclear accumulation of β-catenin and in so doing enhance thyroid epithelial cell proliferation in a Wnt-independent manner. IGF-1R appears to play an indispensable role in specific aspects of TSHR signaling. In mice where tissue-specific IGF-1R expression was knocked down in the thyroid, TSH levels were increased while thyroxine levels were decreased[53]. On the other hand, over-expressing IGF-1R in thyroid reduces the TSH concentration requirement for regulating the synthesis of thyroid hormones[54]. Tsui *et al*[55] demonstrated that IGF-1R and TSHR form a physical complex in TAO orbital fibroblasts and thyroid epithelial cells. Interrupting IGF-1R-dependent signaling with an IGF-1R blocking antibody could attenuate the actions of TSH and TSI on post-TSHR signaling. Further, the report contained evidence for TSHR/IGF-1R co-localization as assessed by confocal microscopy and through co-precipitation pull-down studies. Dependence on IGF-1R for TSHR-initiated signaling in orbital fibroblasts has been confirmed more recently by another laboratory group[34]. The potential involvement of IGF-1R and activating antibodies against it in TAO has provoked lively discussions. While strong opinions have been voiced[56], essentially nothing is currently known about the relative contributions of either receptor to the pathophysiological events occurring *in vivo*. Thus determination of the merits of either side of the debate will necessarily await further study.

Reconciling the detection of low-level thyroid antigens in the orbit

Among the first reports suggesting that proteins thought to be restricted to thyroid could also be detected in the orbit was that of Kriss[57]. He proposed that thyroid antigens such as thyroglobulin (Tg) might translocate from the thyroid to the orbit in TAO. He employed thyroidolymphography to examine lymphatic drainage in hyperthyroid patients with GD.^{99}mTc Sulfur colloid injected directly into the hyperplastic thyroids of these patients exhibited rapid movement into their lymphatics. This same group successfully immunized mice with human extra-ocular muscle[58]. This resulted in the generation of anti-Tg antibodies. Subsequently, Marino and colleagues[59] have characterized Tg in orbital contents. They detected intact Tg in tissues from individuals with TAO and could identify T_4 in the material. This led them to conclude that the Tg most likely originated in the thyroid and somehow translocated to the orbit. The protein was undetectable in tissues from healthy donors. This same group found that Tg in orbital tissues formed a complex with glycosaminoglycans[60]. Further, they found a potential relationship between orbital Tg content and previous treatment with radioactive iodine[61].

Orbital fibroblasts exhibit exaggerated responses to inflammatory mediators

Among the properties that appear to distinguish orbital fibroblasts from those inhabiting other anatomic regions are the repertoire of proteins they express[62] and their particularly

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robust responses to inflammatory cytokines. The magnitude of these responses appears to set these cells apart from other fibroblasts. Evidence suggests inherent differences between orbital fibroblasts from donors with TAO and those from healthy individuals[63]. In addition, external molecular cues derived from infiltrating mononuclear cells may play important roles in fibroblast behavior *in situ*[64,65]. Among these are responses to members of the IL-1 cytokine family. In particular IL-1β frequently elicits dramatic inductions of genes such as those encoding other inflammation-related proteins, including IL-6 and IL-8[66,67] chemokines and chemoatractants (IL-16, RANTES)[39], and enzymes involved in the synthesis of immune mediators of inflammation[68,69]. Notable enzymes which are expressed and differentially induced in TAO orbital fibroblasts include 15-lipoxygenase[68] and prostaglandin endoperoxide H synthase-2[69] (PGHS-2, AKA COX-2, the inflammatory cyclooxygenase) and their production of 15-HETE and prostaglandin E_2, respectively. A peculiar profile of IL-1 receptor antagonist (IL-1RA) isoforms appears to be among the factors underlying these exaggerated responses to cytokines. These patterns of expression diverge from those identified in fibroblasts from other connective tissue depots[70]. Of particular note is the relatively low level of secreted IL-1RA (sIL-1RA) generated by orbital fibroblasts in response to multiple stimuli. sIL-1RA acts as a dominant modulator of IL-1 actions[71] . Therefore, its low levels renders orbital fibroblasts particularly susceptible to the actions of IL-1β. In contrast, intracellular IL-1RA (icIL-1RA) is highly expressed by these cells. While this isoform attenuates responses downstream from IL-1 receptor, it does not block the actions of exogenous IL-1β[72]. IL-1RA expression is provoked by several factors in these cells, including IL-1β and TSH[70,73]. A very recent report has disclosed the modulatory influence of phosphatase and tensin homologue (PTEN) on IL-1RA expression by fibroblasts as well as that of IL-1α and IL-1β in response to TSH[74]. Thus the array of inflammatory genes as well as their modulators expressed in orbital connective tissues may determine the magnitude and duration of immune reactivity in TAO.

Orbital fibroblasts produce hyaluronan and undergo adipogenesis in TAO

The propensity of soft tissues to expand in TAO is a consequence, at least in part, of an accumulation of HA. This is associated with increased *de novo* adipogenesis, resulting in increased fat volume. Orbital fibroblasts express the molecular machinery necessary for HA synthesis, including UDP-glucose dehydrogenase[75,76] and the terminal hyaluronan synthases, of which three have been identified[77,78]. When activated by cytokines such as IL-1β, CD40 ligand, leukoregulin, prostaglandin D_2, or growth factors such as IGF-1 and bTSH, fibroblasts express levels of these enzymes that result in accelerated HA production[33,79–82]. It is currently uncertain whether the apparent accumulation of HA in the TAO orbit results from altered rates of synthesis or delayed macromolecular degradation. Because HA chain length is an important determinant of its biological impact[83], a systematic analysis of HA size and determination of whether this becomes skewed in TAO remain important voids in our current understanding. Further, the relative abundance of other glycosaminoglycans, such as chondroitin sulfate, heparan sulfate, and dermatan sulfate, has yet to be quantified.

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Characterization of orbital connective tissue reveals multiple cell types: emerging evidence for orbital fibroblast heterogeneity

Identifying substantial differences in the behavior *in vitro* of orbital fibroblasts from those found in other tissues has prompted a thorough examination of their distinguishing phenotypic attributes, including their embryonic lineages. Determination of the derivation of orbital connective tissue can be traced to the work of Noden[84] who called attention to the origins of its components, fate, and interactions with epithelial elements. Among its peculiar attributes, orbital fat comprises a heterogeneous population of fibroblast-like cells, especially in TAO. Fibroblasts derived from this fat can be divided into discrete populations based upon display of cell surface molecules[85]. Despite sharing similar morphologies[86] and ultrastructure[87] in culture, these cells exhibit distinct properties[85,88–92]. Fibroblasts from healthy orbital connective tissues as well as those from individuals with TAO can be bisected into two subsets based on the display of CD90 (akaThy-1), a membrane spanning glycoprotein. Thy-1^{+} and Thy-1^{-} subsets exhibited divergent patterns of terminal differentiation. The former expresses smooth muscle-specific actin and develops into myofibroblasts when treated with TGF-β. In contrast, Thy-1^{-} fibroblasts express high levels of peroxisome proliferator-activated receptor γ (PPARγ) and respond to thiazolidinediones by accumulating cytoplasmic lipid droplets and expressing lipogenic enzymes. Differences in these cell subsets were characterized recently by Li *et al* using a 3-dimensional model[92]. Responses to cytokines such as IL-1β appear to differ in the two subsets. Subsequent studies by Lehmann *et al*[93] have demonstrated recently a molecular interplay between Thy-1^{+} and Thy-1^{-} orbital fibroblasts. They found that Thy-1^{+} orbital fibroblasts secrete a soluble factor which seems to prevent cells of that phenotype from differentiating into adipocytes. The actions of this inhibitory factor may be mediated by altering the binding of PPAR-γ to its DNA binding site. Thus it is possible that the balance between Thy-1^{+} and Thy-1^{-} fibroblasts might determine whether the orbital tissues undergo adipogenic differentiation or fibrosis. Further investigation into the relationship between these orbital fibroblast subsets, including better definition of their phenotypic attributes and biosynthetic repertoires, should yield fundamental insights into the biology of tissue changes associated with TAO.

Identifying infiltrating CD34^{+} fibrocyte in the orbit allows reconciliation of several unexplained tissue characteristics associated with TAO

Fibrocytes are monocyte linage progenitors from the bone marrow that have been implicated in many aspects of wound repair, tissue remodeling, and immune function[94,95]. They have been associated with several diseases and repair processes including thermal burns[96], glomerular disease[97], hepatic fibrosis[98], asthma[99], and rheumatoid arthritis[100]. They are identified by a specific pattern of protein expression and display of cell surface markers[101]. These include CD45, CXCR4, CD34, and the production of collagen I. Fibrocytes can terminally differentiate into either myofibroblasts or adipocytes depending on the molecular environment to which they are subjected[102]. Specifically, when exposed to PPARγ, they accumulate triglycerides and differentiate into mature adipocytes. In contrast, when activated through the Smad pathway following treatment *in vitro* with TGF-β, they differentiate into myofibroblasts and express high levels of α-smooth muscle specific actin.

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Fibrocytes play roles in immune function, including their ability to present antigens through MHC II to T cells[103]. These cells are extremely rare in the circulation of healthy individuals, where they account for less than one percent of mononuclear cells. Their abundance increases dramatically in certain pathological states, such as those associated with extensive acute or chronic tissue injury[104,105]. This appears to be the case in thermal injury where the cells not only circulate in greater abundance but infiltrate affected tissue[106]. This homing occurs through chemokine activities. Animal-based studies have implicated the CXCR4/CXCL12 chemokine network as an important localization determinant involved in the pathogenesis of lung fibrosis[107].

Douglas *et al*[10] have reported that the frequency of circulating $CD34^+$ fibrocytes is substantially increased in GD (Fig. 1). These cells were detected *in situ* in TAO orbital fat but appear to be absent in tissues from healthy individuals (Fig. 2). The authors identified an abundant subset of TAO orbital fibroblasts displaying a $CD34^+CD31^-CXCR4^+$ phenotype, suggesting that they represent fibrocytes that had infiltrated orbital tissues and transitioned to $CD34^+$ fibroblasts. The cells no longer express CD45 but produce collagen I. They reside in a cellular context which includes $CD34^-$ fibroblasts in the TAO orbit. $CD34^-$ fibroblasts are believed to be the identical fibroblasts that are uniformly identified in healthy tissues but that have been partially replaced in TAO. Since both fat expansion and fibrosis represent important components of tissue remodeling associated with TAO[108], fibrocytes are now considered prime candidates for therapeutic targeting.

Among the attributes recently ascribed to fibrocytes is a high level of TSHR mRNA expression that is unprecedented in the extra-thyroidal compartment[10]. Steady-state abundance of the transcript is considerably lower than that found in thyroid tissue but levels of the protein appear to be equivalent on fibrocytes and thyroid epithelial cells and substantially higher than those on orbital fibroblasts (Fig. 3)[55]. Its presence on fibrocytes suggests that the receptor might mediate actions of TSH and TSI locally in the connective tissue compartment[7]. Several reports have appeared documenting responses of fibrocytes to TSH and M22, a monoclonal TSI. These have demonstrated increases in cytokine production, including IL-1β, IL-1RA, TNF-α, IL-6, IL-8, and MCP[8,30,73,109] (Fig. 4). These responses appear to be qualitatively similar in cells from patients with TAO and healthy individuals but the magnitude of response appears greater in the former[106]. In contrast to fibrocytes, TAO orbital fibroblasts exhibit substantially less robust responses to TSH. This divergence in the magnitude of response might be attributed to higher TSHR levels in fibrocytes and qualitative differences in the relevant signaling pathways.

Besides TSHR, other “thyroid specific” proteins, such as Tg[110], sodium iodide symporter (NIS), and thyroid peroxidase (TPO) are also expressed by cultured fibrocytes[111]. Levels of Tg as well as these other proteins are substantially lower than those found in thyroid but are still readily detectable using standard techniques. Fibrocyte-expressed Tg can incorporate iodine, a process that appears to be TSH-independent. As with TSHR, Tg, NIS, and TPO mRNAs are considerably less abundant in TAO orbital fibroblasts than in cultured fibrocytes and the respective proteins are expressed at the limits of detection. It is thus possible that the expression of TSHR and Tg by infiltrating fibrocytes could account for the low levels of both proteins detected in TAO orbital tissues[21.57–61]. In addition to those

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proteins associated with thyroid function and disease, fibrocytes express autoantigens associated with type 1 diabetes mellitus[112]. Of potential importance is the identification of autoimmune regulator protein (AIRE), a non-traditional transcription factor[113], as an essential participant in the expression by fibrocytes of these 4 proteins[111]. Knocking down AIRE expression substantially reduces the levels of all 4 thyroid proteins (Fig. 5). Further, interrogation of TSHR, Tg, NIS, and TPO expression in a patient with loss of function AIRE mutation reveals a similar reduction (Fig. 6).

Recent studies have disclosed interactions between native ($CD34^-$) orbital fibroblasts and $CD34^+$ orbital fibroblasts which are putatively derived from $CD34^+$ fibrocytes. It appears that signals from $CD34^-$ fibroblasts may repress the expression of thyroid proteins, thereby accounting for their lower levels as fibrocytes transition to $CD34^+$ orbital fibroblasts[110,111]. Supporting this possibility are studies revealing that parental orbital fibroblast strains, comprising a mixture of $CD34^+$ and $CD34^-$ cells and subjected to cytometric cell sorting into pure subsets on the basis of CD34 display yield dramatically higher thyroid protein expression in the $CD34^+$ subset (Fig. 7). These findings carry substantial implications for the potential mechanisms through which aspects of $CD34^+$ fibroblast phenotype might be modulated by $CD34^-$ fibroblasts. The expression repertoire of antigens by fibrocytes infiltrating the orbit in TAO therefore might be governed by the capacity of residential $CD34^-$ fibroblasts to modulate their magnitude and diversity. In so doing, they might help determine the pathogenic immune responses. **Could insinuation of fibrocytes into TAO reveal attractive therapeutic targets?**

In our view, identifying relatively high-level expression of functional TSHR in fibrocytes and the apparent infiltration of these cells into the TAO orbit might be exploited therapeutically. Strategies for interrupting TSHR signaling are being developed and the properties of these agents appear promising as potential drugs. Highly selective small molecule antagonists of TSHR have been reported by Neumann *et al*[114] and among the latest is an agent designated ANTAG3 which demonstrates activity *in vitro* as well as in mice. Alternatively, stimulatory and blocking antibodies targeting TSHR have been developed[115,116] and their divergent interactions with the receptor have been scrutinized in great detail[117]. Either approach might prove effective in reducing the impact of the inflammatory phenotype exhibited by TSH-engaged fibrocytes. Alternatively, targeting IGF-1R signaling with a blocking antibody such as teprotumamab has been shown to substantially reduce TSHR-mediated cytokine expression. That blocking antibody substantially reduced the induction by TSH of IL-6 in fibrocytes[118], strongly suggesting that the IGF-1R pathway in these cells might be interrupted as another therapeutic approach. As yet untested but worthy of investigation are strategies that interfere with fibrocyte infiltration, such as those interrupting relevant chemokine pathways. It would appear that emerging insights into the biology of the orbit will yield more effective treatments of TAO.

Conclusion

Bone derived fibrocytes have recently been identified as becoming more numerous in GD and TAO. They appear to infiltrate the orbit in TAO where they putatively transition into a subset of orbital fibroblasts. Fibrocytes express relatively high levels of functional TSHR

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through which TSH and TSIs provoke expression of an array of inflammatory genes including those encoding cytokines. It would appear that TSHR signaling in fibrocytes and orbital fibroblasts is dependent, at least partially on functional IGF-1R. Taken together, these recent findings insinuate fibrocytes in the pathogenesis of TAO. When considering this evidence, it is imperative that the reader consider that virtually all of these findings come from a single research laboratory. This work must therefore be independently corroborated by others and expanded to in vivo models before any firm conclusions can be drawn.

Acknowledgments

The author gratefully acknowledges the great editorial assistance of Ms. Linda Polonsky and the help of Ms. Justyna Piernicka in the preparation of this manuscript. This work was supported in part by National Institutes of Health grant EY08976, Center for Vision grant EY007003 from the National Eye Institute, and unrestricted grants from Research to Prevent Blindness, and the Bell Charitable Foundation.

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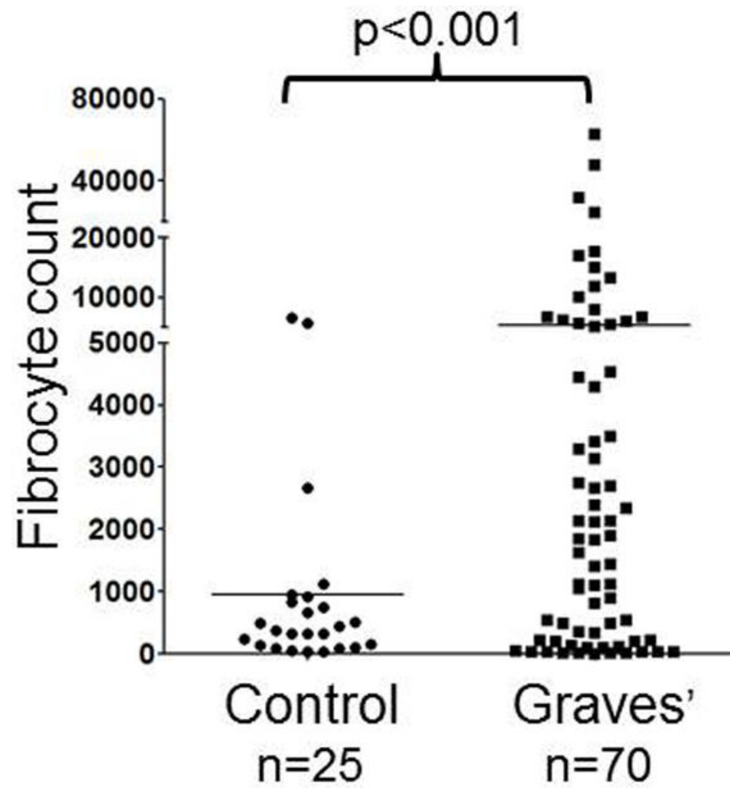

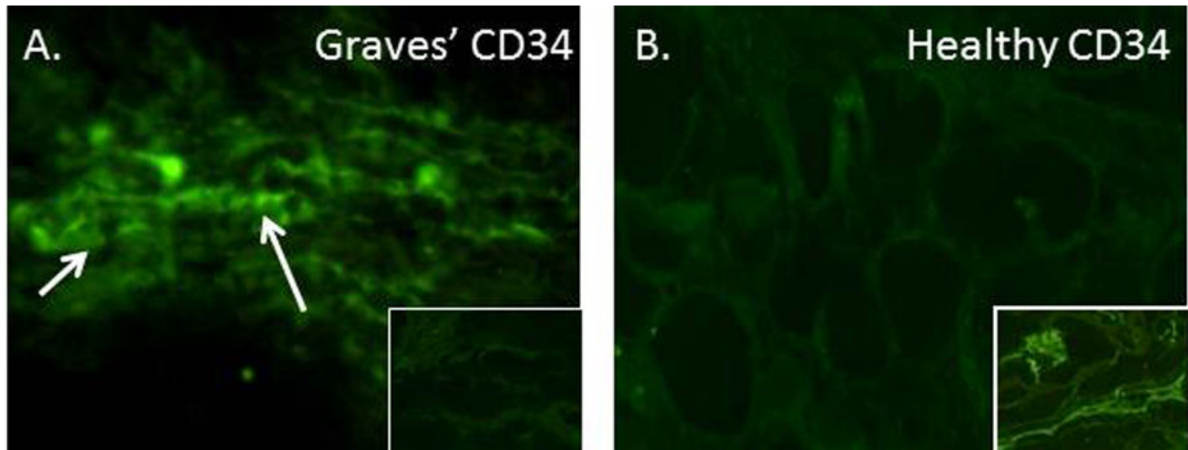

Figure 1.

Increased frequency of CD34$^+$ fibrocyte generation from the PBMCs of 70 patients with GD compared with 25 healthy control donors. PBMCs were inoculated at a density of 5×10^6 cells/well. Cultures were incubated for 14d. Adherent cells (<5% of starting cells) were collected and counted. (Reprinted with permission; Douglas *et al.* Increased generation of fibrocytes in thyroid-associated ophthalmopathy, Reference 6, Copyright 2010. The Endocrine Society.)

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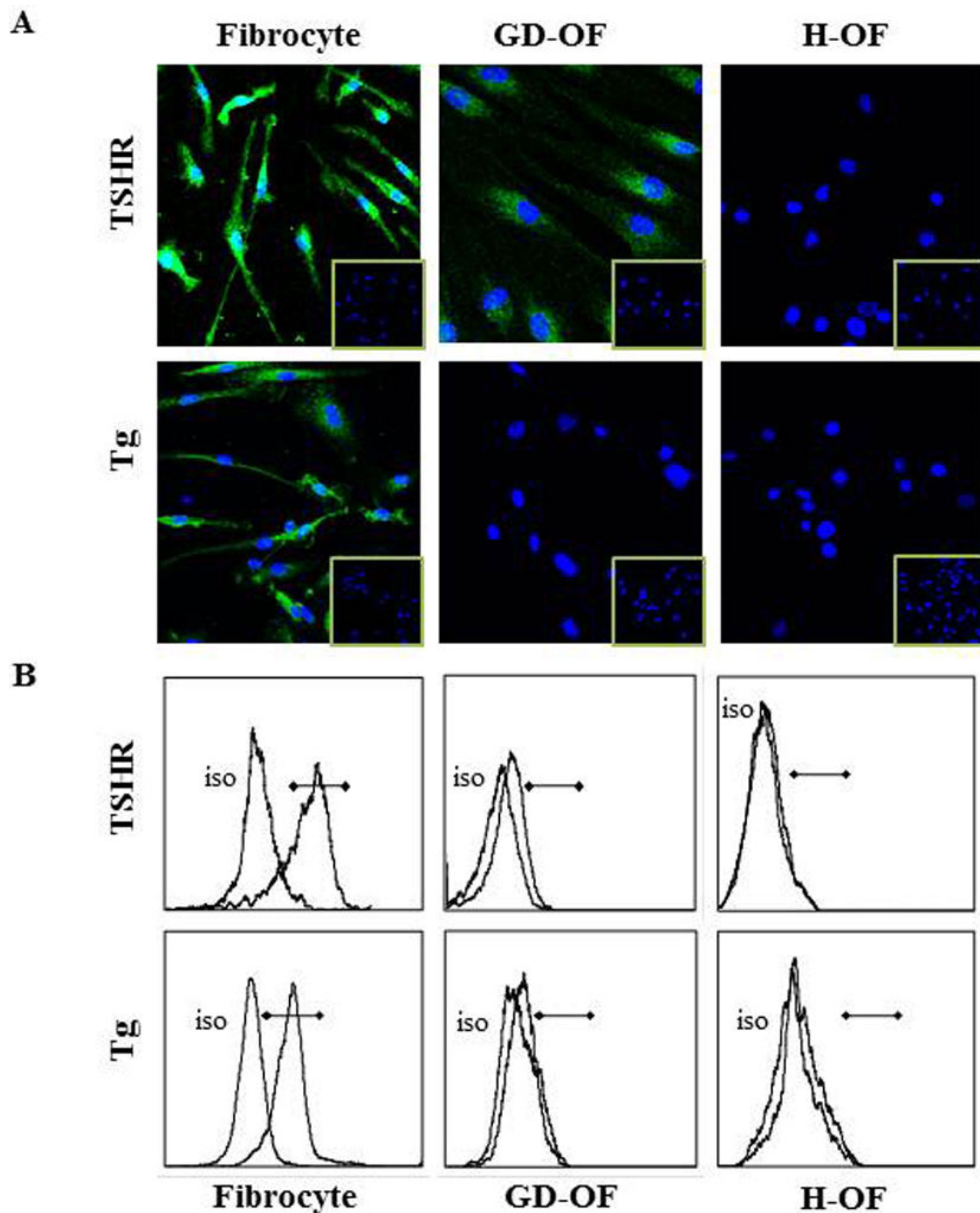

Figure 2.

CD34+ LSP-1+ TSHR+ fibrocytes can be identified in the orbital tissue of patients with TAO but are absent in tissues from healthy donors. A, CD34 expression (arrows, green fluorescein isothiocyanate) in TAO- derived tissue (inset, negative control staining). B, Absent, CD34 expression in healthy tissue (inset, positive control). C, LSP-1 expression in TAO-derived tissue [red, arrows, nuclei counter-stained with 4′,6′ –diamino-2-phenylindole (DAPI)(blue)(inset, negative, control). D, Absence of LSP-1 expression in healthy tissue (inset, negative controls). E, CD31 expression in disease-derived tissue is limited to vascular endothelium (red, arrows). F, Hematoxylin and eosin-stained consecutive thin sections of

the same orbital tissue (×40). G, Fibrocytes present in orbital tissue from patients with TAO co-express CD34 and TSHR. Thin-sectioned tissue from a donor with TAO was stained with anti-CD34 (green) and anti-TSHR (red) antibodies. Nuclei were counterstained with DAPI (blue). Thin sections were then subjected to confocal microscopy. *Inset* contains a negative staining control. (Reprinted with permission; Douglas *et al.* Increased generation of fibrocytes in thyroid-associated ophthalmopathy, Reference 6, Copyright 2010. The Endocrine Society.)

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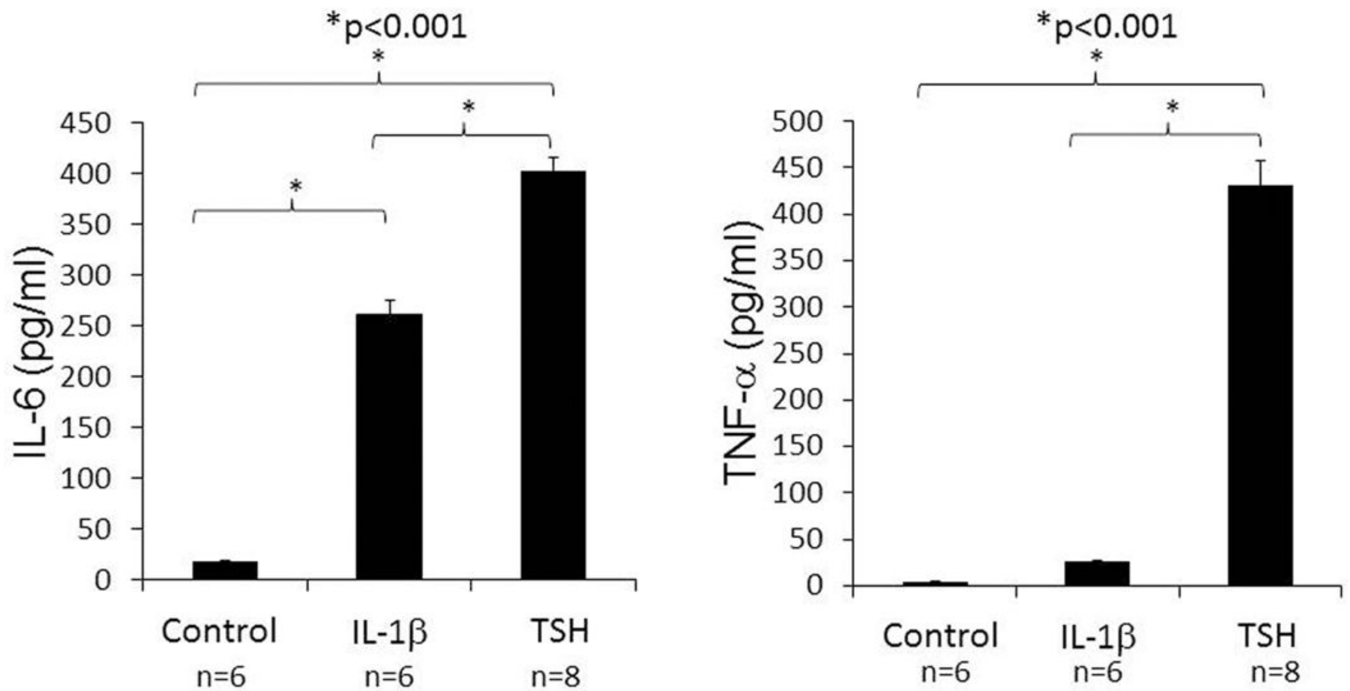

Figure 3.

Analysis of TSHR and Tg expression by human fibrocytes and fibroblasts by (A) immunofluorescence staining and (B) flow cytometry. Cultivated cells were (A) fixed, blocked, and stained with either anti-TSHR, anti-Tg, or isotype control mouse IgG followed by Alexa Fluor 488-conjugated goat anti-mouse Abs. Nuclei were stained with DAPI, and thin sections were subjected to confocal fluorescence microscopy. (Insets) Isotype IgG staining. (B) Fibrocytes and fibroblasts were incubated with phycoerythrin-conjugated anti-TSHR, anti-TG, or isotype mAbs and Alexa Fluor 488 goat anti-mouse IgG. They were subjected to flow cytometry. Horizontal lines denote fluorescence intensity compared with isotype controls. (Reprinted from; Fernando *et al*. Human fibrocytes co-express thyroglobulin and thyrotropin receptor, Proceedings of the National Academy of Sciences, Reference 103, Copyright 2012.)

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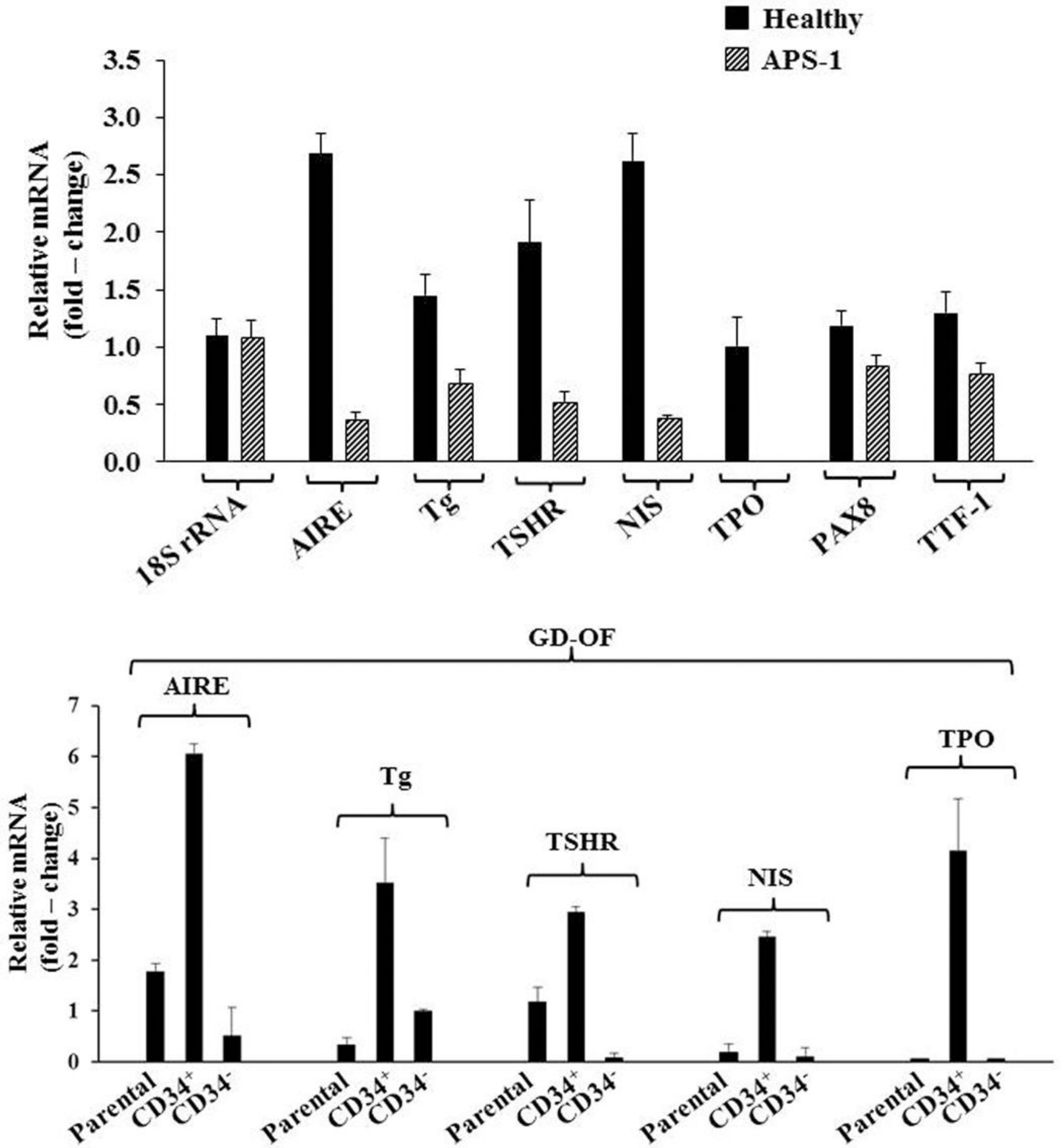

Figure 4.

TSHR displayed on fibrocytes generated from PBMCs can initiate cytokine production. Cultures cells from a patient with GD were treated with bTSH (5 mU/ml) or IL-1β (10 ng/ml) for 48h. Medium was subjected to ELISAs specific for IL-6 (left panel) or TNF- α (right panel). Data are expressed as the mean ± SEM of three replicate culture wells. *, P <

0.001. (Reprinted with permission; Douglas *et al.* Increased generation of fibrocytes in thyroid-associated ophthalmopathy, Reference 6, Copyright 2010. The Endocrine Society.)

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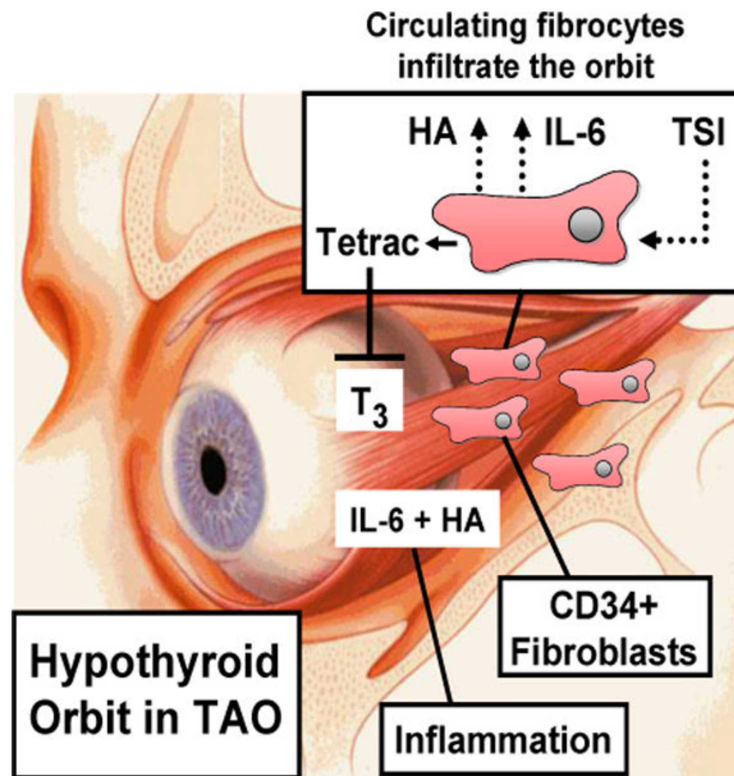

Figure 5.

Interfering with AIRE expression knocked-down levels of AIRE, Tg, TSHR, NIS, TPO, PAX8, and TTF-1 but not PGHS-2 or 18S RNA. A, Fibrocytes from four different donors were transfected, either with scrambled (control) siRNA or siRNA directed against AIRE. Cultures were then incubated for 48 hours, and RNA was harvested and then subjected to real-time PCR. Data are expressed as mean ± SD of three independent determinants. B, Fibrocyte cultures were transfected with either siRNA or siRNA targeting AIRE for 96 hours. Tg protein was quantified by labeling with [35S]methionine (40μCi/mL) and then immunoprecipitated. TSHR and NIS were quantified by flow cytometry. Levels of TPO were assessed by Western blotting. (Reprinted with permission; Fernando *et al.* Expression of thyrotropin receptor, thyroglobulin, sodium iodide symporter, and thyroperoxidase by fibrocytes depends on AIRE, Reference 104, Copyright 2014. The Endocrine Society.)