Mitophagy defects exacerbate inflammation and aberrant proliferation in lymphocytic thyroiditis

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Research Article

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Abstract

Background

Mitochondrial dysfunction of the thyroid due to defective mitophagy has been observed in lymphocytic thyroiditis (LT). However, the effect of impaired mitophagy on the pathogenesis of LT has not been elucidated.

Results

We investigated the molecular pathological effect of mitophagy defects in thyroid glands through bioinformatics and histological approach using human and mouse thyroids and human thyroid cells. In this current study, it is showed that PINK1, a key regulator of mitophagy, is compromised in human thyroids with LT, and inflammatory responses and nodular hyperplasia are induced in the thyroids of PINK1-deficient mice. We found that mitophagy defects trigger pro-inflammatory cytokine production in thyroid cells and immune cell recruitment. Additionally, mitochondrial reactive oxygen species-driven hypoxia depletes CREB, a transcriptional repressor of amphiregulin (AREG), resulting in aberrant thyroid cell proliferation by AREG-mediated epidermal growth factor receptor signaling activation.

Conclusions

This signaling pathway could be a potential therapeutic target for thyroid goitrous changes in patients with LT. Our findings reveal the mitophagy defects in the thyroid that may be involved in LT pathogenesis and progression.

1. Background

Mitochondrial dysfunction has been found to play a critical role in the pathogenesis of various diseases, including metabolic, cardiovascular, neurodegenerative, and neuromuscular diseases[1]. Mitophagy, a mitochondrial quality and quantity control mechanism whereby damaged or superfluous mitochondria are eliminated, is key to maintaining cellular homeostasis, and defects in mitophagy contribute to excessive oxidative stress[2, 3]. The thyroid gland is an endocrine organ with a high energy demand, in which oxidative processes are essential for thyroid hormone synthesis. As reactive oxygen species (ROS) are required in the initial stages of thyroid hormone production—during iodide oxidation by thyroid peroxidase (TPO)—the thyroid is particularly susceptible to oxidative damage[4, 5]. Therefore, mitochondrial quality control for regulating ROS accumulation is crucial in thyroid cells, and mitophagy defects can lead to thyroid diseases[6].

Chronic lymphocytic thyroiditis (LT), also known as Hashimoto’s thyroiditis, is the most common cause of hypothyroidism in iodine-sufficient areas of the world, and affects up to 10% of the global population[7].
LT is characterized by the infiltration of inflammatory cells in the thyroid gland and the production of autoantibodies to thyroid-specific antigens such as TPO and thyroglobulin[8, 9]. Although the clinical presentation of LT may vary, it typically manifests as a gradual loss of thyroid function and nodular goiter formation with disease progression[10, 11, 12]. The etiology of LT is multifactorial, and the mechanisms underlying the induction of immune cell recruitment and goitrous thyroid enlargement are not fully understood.

There is evidence of mitophagy defects in thyroid cells with LT, with a marked increase in the abundance of dysfunctional mitochondria with a swollen appearance and irregular cristae[13, 14, 15], suppressed autophagosome levels[16], and excessive oxidative stress and mitochondrial ROS (mtROS)[17]. Moreover, oncocytic change, characterized by cellular enlargement with abundant oxyphilic granular cytoplasm due to the accumulation of abnormal mitochondria, in thyroid follicular epithelial cells is a characteristic feature of LT[18]. However, the effect of impaired mitochondrial clearance on LT pathogenesis has not been elucidated.

In this study, we investigated the role of mitophagy defects in the thyroid, which might be involved in LT pathogenesis and progression, using transcriptomic and experimental approaches. Our findings may aid in the development of a promising new therapeutic strategy for protection against the advancement of LT.

2. Results

2.1. Downregulation of **PINK1** was associated with LT and immune-related genes in the human thyroid

To investigate the changes in the expression of genes regulating mitophagy in LT, we used RNA sequencing data and histological information on human thyroid tissues (total, n = 653; no LT, n = 589; LT, n = 64) from the GTEx database. Of the top 10 mitophagy-related genes with the highest relevance score obtained from GeneCards[19], **PINK1** was the most significantly downregulated gene in thyroids with LT compared with those without LT (Fig. 1A, Table S1). Subsequently, to further explore the biological role of **PINK1** in the human thyroid, we divided the samples into low- and high-expression groups using the median value of **PINK1** and obtained DEGs between the two groups. We then performed functional annotation analyses of the KEGG pathways and GO terms using the DEGs upregulated in the low-**PINK1** group. All the top five KEGG pathways and most of the top five GO terms in each biological process and molecular function domain were found to be related to immune processes (Fig. 1B). Additionally, **PINK1** expression was negatively correlated with the total immune cell infiltration score estimated using CIBERSORTx, and similar trends were observed in 6 out of 22 subsets of immune cells, such as follicular helper T cells, CD8+ T cells, regulatory T cells, activated NK cells, naïve B cells, and M1 macrophages (Fig. 1C, Fig. S1), which are highly related to cytotoxicity and immune modulation. Furthermore, when analyzing the protein expression of PINK1 and 8-hydroxy-2’-deoxyguanosine (8-OHdG), an oxidative damage marker of mitochondrial DNA, using IHC in human thyroid tissues with and without LT (n =
8/each), we found that the cytoplasmic expression of PINK1 was lower (Fig. 1D) and that of 8-OHdG was higher in thyroid cells with LT (Fig. 1E). Additionally, the IHC scores for PINK1 and 8-OHdG were negatively correlated across the samples (Fig. S2). Taken together, PINK1 repression was observed in human thyroids with LT and was highly associated with mitochondrial oxidative damage and immune responses.

### 2.2. Mitophagy defects due to PINK1 loss induced thyroid hyperplasia and inflammation

To determine the effect of mitophagy defects on the thyroid, we used Pink1 KO and WT mice, and 24-month-old mice were used to investigate chronic changes. Analyses of morphological and histological changes in the thyroid revealed goitrous and nodular changes (Fig. 2A) and a significant increase in thyroid weight (Fig. 2B) in the thyroids of Pink1 KO mice; however, no significant increase in serum thyroid-stimulating hormone levels (Fig. 2C) was observed. Histological findings showed that while the normal thyroid follicular cell structure was maintained in Pink1 WT mice (Fig. 2D), distinct histological findings of nodular hyperplasia (red box in Fig. 2D) and an increase in the size of colloid-filled follicles (gray box in Fig. 2D) were observed in the thyroids of Pink1 KO mice. Additionally, evident immune cell infiltration and lymphoid germinal center formation were observed in Pink1 KO mice (pink box in Fig. 2D). Thyroid follicular cells with oncocyctic changes due to aberrant accumulation of mitochondria were observed in the thyroids of Pink1 KO mice (Fig. S3A). Furthermore, molecular markers of oncocyctic changes such as LMP2 expression (Fig. S3B), and interferon gamma signaling[20, 21] scores were significantly increased in Pink1 KO mice (Fig. S3C). These histological and molecular findings were consistently observed in human thyroid tissues with LT (Fig. S3D-F). Subsequently, we used transmission electron microscopy to analyze the ultrastructure of the mitochondria in thyroid cells. The thyroid cells of Pink1 WT mice showed normal mitochondria characterized by intact membrane and cristae (The upper part of Fig. 2E), whereas those of Pink1 KO mice showed accumulation of abnormal mitochondria with swelling and bursting appearance (The lower part of Fig. 2E). Therefore, long-term downregulation of Pink1 in the mouse thyroid resulted in aberrant cell proliferation, immune cell infiltration, and oncocyctic changes with abnormal mitochondrial accumulation in thyroid cells, all of which are comparable to those seen in human chronic LT.

### 2.3 Mitophagy defects due to PINK1 loss upregulated genes involved in immune response and damage-associated molecular patterns (DAMPs) in the thyroid

To explore the molecular mechanisms of phenotypic changes in the thyroid due to PINK1-loss-induced mitophagy defects, RNA sequencing analysis was performed for the thyroid tissues of Pink1 KO and WT mice. We first confirmed that the thyroid glands of the Pink1 KO mice had a deletion of exons 4–7, as previously described[22] (Fig. S4). Subsequently, we performed KEGG pathway and GO enrichment analyses with the DEGs between the thyroids of Pink1 KO and WT mice. Most pathways and GO terms enriched with upregulated DEGs in Pink1 KO mouse thyroids were associated with the activation of the immune response, which accounts for all of the top five KEGG pathways and eight of the top 10 GO terms.
Moreover, the total immune infiltration score (Fig. 3B) and ROS-associated gene signature (Fig. 3C) were significantly higher in the thyroids of Pink1 KO mice than in those of Pink1 WT mice. As dysfunctional mitochondria have been reported to trigger sterile inflammatory reactions by activating DAMP signaling pathways in several previous studies[23, 24], we hypothesized that DAMPs (endogenous molecules released in response to cellular damage that induce potent inflammatory responses as danger signals) can induce the activation of immune responses observed in PINK1-deficient thyroids. To test this hypothesis, we applied the scoring analysis to gene sets of biological pathways related to mitochondrial-derived DAMPs. All the DAMP-related gene set scores were significantly higher in Pink1 KO mouse thyroids than in Pink1 WT mouse thyroids, such as NLR family pyrin domain containing protein 3 (NLRP3) inflammasome, toll-like receptor 9 (TLR9), stimulator of interferon genes (STING), and nuclear factor kappa light chain enhancer of activated B cells (NF-κB) (Fig. 3D). Consistent with the results of the RNA sequencing analysis in PINK1-deficient mouse thyroids, the ROS-related gene score and DAMP-related signaling pathway scores were significantly increased in human thyroids with LT (Fig. S5A-E).

2.4 Mitophagy defects exacerbated inflammation via cytokine production from thyroid cells as well as immune cell recruitment

A human thyroid cell line, HTori-3, was used to obtain insights into the mechanisms of DAMP signaling-related immune responses, and the cells were transfected with PINK1-siRNA or control-siRNA. PINK1 knockdown increased cellular and mtROS production as quantified using H2DCFDA and MitoSOX, respectively (Fig. 3E). The mRNA expression of DAMP signaling-related proinflammatory cytokines such as IL1B, IL6, and TNF in human thyroid cell lysates was upregulated in the presence of PINK1 knockdown (Fig. S5F). The secreted protein levels of these cytokines in the conditioned medium (CM) from HTori-3 cells transfected with PINK1-siRNA (siPINK1-CM) were also higher than those in the CM from HTori-3 cells transfected with control-siRNA (siControl-CM) (Fig. 3F). Subsequently, we examined the effect of Mdivi-1, a mitophagy inhibitor that modulates mtROS production[25]. Consistent with the findings of PINK1 knockdown in HTori-3 cells, Mdivi-1-treated human thyroid cells showed increased expression and secretion of proinflammatory cytokines (IL1B, IL6, and TNF) (Fig. S5G, Fig. 3F). Because immune cell infiltration was prominent in both mouse and human PINK1-deficient thyroid tissues, we determined whether thyroid cells with impaired mitophagy could stimulate immune cell chemotaxis. Interestingly, in the transwell migration assay, the migration potential of Jurkat cells, a human T lymphocyte line, was significantly increased by treatment with siPINK1-CM compared with siControl-CM (Fig. S5H). Taken together, the results show that mitophagy defects in the thyroid induce inflammation by increasing the release of DAMP-related proinflammatory cytokines from the thyroid cell itself, which causes immune cell recruitment to the thyroid.

2.5. Mitophagy defects increased AREG expression and secretion in thyroid cells

Because mitophagy defects caused goitrous changes and hyperplasia of thyroid tissue in Pink1 KO mice (Fig. 2A, B, E), we investigated the mechanism of aberrant cell proliferation. In the GO functional
annotations of RNA sequencing analysis of mouse thyroids, although most of the enriched gene sets were immune-related, a gene set of growth factor activity was significantly enriched with upregulated DEGs in Pink1 KO mice (Fig. 3A). Among the genes of this gene set, only amphiregulin (Areg in mice, AREG in humans), an epidermal growth factor receptor (EGFR) ligand[26, 27], satisfied the criteria for upregulated DEG in human thyroids with LT (Table S2). To verify this, we first performed IHC staining of AREG in human thyroid tissues and demonstrated that thyroid follicular cells in thyroids with LT showed elevated AREG protein expression, whereas those in thyroids without LT did not (Fig. 4A). Additionally, it has been reported that the CREB acts as a transcription repressor by binding to the cAMP response element sequences in the AREG promoter of thyroid cells[28]; moreover, CREB has also been shown to degrade under hypoxia[29, 30]. Therefore, we investigated whether the molecular mechanism of increased AREG expression was associated with a decrease in its transcriptional repressor, CREB. We found that AREG expression was positively correlated with the hypoxia-related gene signature in mouse and human thyroid tissues (Fig. S6A, B). Subsequently, we demonstrated that the protein expression of HIF1A, which is stabilized under hypoxic conditions, increased while CREB decreased in the thyroids of Pink1 KO mice compared to those of WT mice (Fig. S6C, D). In vitro experiments showed that the mRNA expression of AREG was significantly increased by PINK1 knockout and Mdivi-1 treatment in HTorI-3 cells (Fig. 4B), as well as secreted protein levels of AREG in siPINK1-CM and Mdivi-1-treated-CM (Fig. 4C).

2.6. AREG promoted aberrant proliferation in mitophagy-deficient thyroid cells by activating EGFR signaling

We investigated whether increased AREG levels activate the EGFR pathway and lead to subsequent cell proliferation. Notably, the gene set scores of the EGFR pathway and its downstream signaling pathways, such as the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways, and the cell cycle-related gene set score were significantly increased in the thyroid glands of Pink1 KO mice (Fig. 4D). Consistent with the results of RNA sequencing analysis in PINK1-deficient mouse thyroids, the EGFR pathway-related gene set scores were significantly increased in human thyroids with LT (Fig. S7A-D). Furthermore, the proliferation of HTorI-3 human thyroid cells was significantly increased by PINK1 knockdown as well as by Mdivi-1 treatment (Fig. 4E). We also found that HTorI-3 cell proliferation was promoted by treatment with siPINK1-CM (Fig. S7E) as well as AREG itself (Fig. S7F). Finally, to inhibit the effect of AREG-induced EGFR signaling activation, we treated HTorI-3 cells transfected with PINK1-siRNA or control-siRNA with cetuximab, an EGFR inhibitor[31]. The increased proliferation of cells treated with PINK1-siRNA was reduced to a level similar to that of cells treated with control-siRNA by treatment with cetuximab (Fig. 4F). This suggested that EGFR inhibition effectively abrogated the stimulatory effect of impaired mitophagy on thyroid cell proliferation.

3. Discussion

Although many previous studies have shown that thyroid cells in LT contain accumulated abnormal mitochondria, it remains unclear as to how this is related to LT pathogenesis[13, 14, 15]. In this study, we demonstrated that PINK1 downregulation in the human thyroid is associated with LT and immune-related
genes. Using a Pink1 KO mouse model, we first reported the effect of mitophagy defects in the thyroid, which were found to induce inflammation and nodular hyperplasia. In vitro analyses of PINK1-deficient thyroid cells showed that inflammation is caused by increased ROS and cytokine production, as well as immune cell recruitment. Finally, mitophagy defects stimulated aberrant cell proliferation through AREG-induced EGFR signaling activation via CREB downregulation under hypoxia.

The PINK1-mediated mitophagy pathway is a key process for the selective elimination of damaged mitochondria, which show disruptive membrane potential[32]. Mitophagy is initiated by the accumulation of PINK1 following depolarization of damaged mitochondria. PINK1 promotes parkin recruitment and the subsequent ubiquitination of outer mitochondrial membrane proteins. The ubiquitinated mitochondria are engulfed by the autophagosome, which fuses with the lysosome and is subsequently degraded. Although mutations in PINK1 have been linked to early onset Parkinson’s disease, PINK1-mediated mitophagy has been studied in many organs to understand the mechanism and effect of aberrant mitochondrial dynamics[22, 33, 34, 35].

Our findings showed that impaired mitophagy causes the accumulation of dysfunctional mitochondria in thyroid cells and triggers excessive mtROS production. mtROS oxidize mitochondrial DNA, which recognized by the DAMP sensors NLRP3 and TLR9 signals. The activated DAMP signaling pathway upregulates the expression of proinflammatory cytokines, such as IL1B, IL6, and TNF, which trigger immune cell infiltration[36, 37, 38, 39, 40]. Mitochondrial dysfunction due to defects in PINK1- and parkin-mediated mitophagy has been demonstrated to trigger inflammation via the STING pathway[41], which is activated by DAMPs. A previous study reported that excess ROS in thyroid cells triggers proinflammatory cytokine secretion through the NF-κB-NLRP3 pathway activated by endogenous DAMPs[42]. Consistent with the results of previous studies, the thyroid of Pink1 KO mice showed significantly upregulated NLRP3, TLR9, STING, and NF-κB signaling pathway scores. Moreover, secretion of proinflammatory cytokines such as IL1B, IL6, and TNF was increased, and migration of human T cells was enhanced in siPINK1-transfected thyroid cells in vitro. Taken together, the results indicate that impaired mitophagy-induced inflammation by excessive mtROS and DAMPs may be one of the etiologies of LT.

Furthermore, we found abnormal cell proliferation in mitophagy-impaired thyroid tissues. Excessive mtROS are well-known hypoxia mediators[43, 44] that stimulate hypoxia-induced transcription[43] and stabilize HIF1A[45, 46, 47, 48]. CREB, a transcriptional repressor of AREG, has been reported to decrease under hypoxia[29, 30]. We showed increased HIF1A and decreased CREB protein expression in Pink1 KO mouse thyroids. We also demonstrated that AREG RNA expression and protein production increased in thyroid tissues and cells with defective mitophagy. Moreover, EGFR signaling pathways, including the PI3K-AKT-mTOR signaling pathway, the main downstream pathway, were activated by increased AREG levels. Activation of the mTOR pathway has been demonstrated to not only trigger cell growth, but also regulate autophagy and mitophagy initiation[49, 50]. We speculate that upregulation of the mTOR pathway via EGFR-EGFR ligand interaction suppresses the mitophagic machinery, resulting in a vicious cycle during the chronic disease course of LT, although further studies are needed to clarify this. Therefore, activation of the EGFR pathway by increased secretion of AREG from thyroid cells via hypoxia-
induced decrease in CREB may be a potential mechanism of aberrant cell proliferation resulting in thyroid goiter in chronic LT.

The goitrous change in the thyroid can be stimulated by elevated thyroid-stimulating hormone levels in chronic LT with hypothyroidism[51]. However, even if normal thyroid function is maintained, nodular goiter formation with chronic inflammation can develop as LT progresses[52]. This can cause cosmetic or obstructive symptoms that may require surgery. Our results showed that the blockage of activated EGFR signaling pathways normalized the aberrant proliferation of mitophagy-impaired thyroid cells. Therefore, targeting the EGFR pathway or AREG activity can be a therapeutic option for thyroid goitrous changes in LT for which there is no effective treatment other than thyroidectomy. Further preclinical studies are required to validate these results.

4. Conclusions

Our study provides a mechanistic understanding of the role of PINK1-mediated mitophagy in LT progression. We discovered that a decrease in PINK1 expression leads to defective mitophagy in LT, resulting in increased mtROS. mtROS trigger DAMP-induced inflammation and cellular proliferation via hypoxia-mediated CREB degradation. Furthermore, our findings suggest that the autocrine activity of upregulated AREG, which turns on the EGFR pathway, is a putative driver of nodular goiter formation in LT. Therefore, investigating strategies for promoting mitophagy may be a promising approach to eliminate damaged mitochondria and excessive mtROS while simultaneously protecting against LT progression. Additionally, blocking AREG or EGFR signaling may be a potential therapeutic target for nodular goitrous changes in LT.

5. Methods

5.1. Human thyroid RNA sequencing data

The human thyroid mRNA expression and clinical phenotype annotation data were downloaded from the Genotype-Tissue Expression (GTEx) v8 (https://www.gtexportal.org/home/) and were used to compare the thyroid with or without LT. This research was approved by the Institutional Review Board of CHA Bundang Medical Center (No. 2022-03-061).

5.2. Mice

PTEN-induced putative protein kinase 1 (PINK1) knock-out (KO) mice were donated by Dr. Xiaoxi Zhuang (Department of Neurobiology, The University of Chicago, Chicago, IL, USA). The mice were backcrossed onto the C57BL/6 background for 20 generations. Pink1 wild-type (WT) mice served as controls. The animal procedures were approved by the CHA University Animal Care and Use Committee (No. 200012).

5.3. mRNA sequencing and data processing
Total RNA was extracted from dissected mouse thyroids using NucleoZOL (Macherey Nagel, Düren, Germany) following the manufacturer's instructions. An RNA library was constructed using the TruSeq Stranded mRNA LT Sample Prep Kit and sequenced on an Illumina TruSeq. The data were processed to remove the remaining adapter sequences and reads were trimmed using Trimmomatic\[53\]. STAR aligner\[54\] was used to align the pre-processed mRNA fastq files to the mouse reference genome (Ensembl/GRCm38). Each gene count was quantified using RSEM\[55\] with the default parameters.

### 5.4. Differentially expressed gene (DEG) analysis and functional enrichment analysis

DEGs were analyzed using the DESeq2 R package\[56\]. The final genes were filtered using the criteria of $|\log_2\text{fold change}| > 1$ (calculated via the effect size shrinkage method of DESeq2) and false discovery rate (FDR) < 0.05, and were used for functional enrichment analysis. Functionally enriched pathways (Kyoto Encyclopedia of Genes and Genomes (KEGG)\[57\] pathways and Gene Ontology (GO) terms\[58\]) were identified using DAVID\[59\] and filtered using the following criteria (population hit > 10, FDR < 0.05).

### 5.5. Immune cell infiltration score and gene set score

In human and mouse RNA expression data, the immune cell infiltration score was calculated using CIBERSORTx\[62\] using the TPM values of each RNA expression dataset and species-specific immune cell gene signature matrix. We used the LM22 (CIBERSORTx default setting) signature matrix for the human immune score and the ImmuCC\[63\] mouse immune cell signature matrix for the mouse immune cell score. The correlation between immune cell score and PINK1 expression was estimated using Pearson's correlation, and six significantly correlated immune subsets were identified using the criteria of $P<0.05$, $|R| \geq 0.2$.

The gene sets were obtained from the Molecular signature database (MsigDB) or the KEGG database. The gene set score calculation method was applied using the same equation for thyroid differentiation score calculation described in the Cancer Genome Atlas study\[64\].

**Gene score** = Mean of median-centered regularized log across genes

The gene set list is described in Supplementary Materials and Methods.

### 5.6. Reagents and antibodies

The siRNAs for PINK1 (sc44598) and control (sc37007) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mitochondrial division inhibitor 1 (Mdivi-1) was purchased from Sigma-Aldrich (M0199; Saint Louis, MO, USA). Recombinant human amphiregulin (AREG, 262-AR-100) was purchased from R&D Systems (Minneapolis, MN, USA). Cetuximab (5 mg/ml) used was Erbitux (C225; Sigma-Aldrich, Saint Louis, MO, USA).
The following primary antibodies for western blotting and IHC were obtained: rabbit anti-PINK1 at a concentration of 1:1000 (ab23707; Abcam, Cambridge, UK), rabbit anti- hypoxia-inducible factor-1α (HIF1A) at 1:1000 (ab179483; Abcam), rabbit anti- cAMP response element binding protein (CREB) at 1:1000 (ab32515; Abcam), goat anti-AREG at 1:1000 (AF262; R&D Systems), and CREB monoclonal antibody at 1:1000 (LB9; Thermo Fisher Scientific).

5.7. Histological analysis and Western blot

For immunohistochemistry (IHC), formalin-fixed paraffin-embedded tissues of human thyroid glands with and without LT were used to validate PINK1 and AREG expression and 8-OHdG damage levels. Immunostaining scores were analyzed according to staining intensity (0, no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining) and extent of the stained area (0 = 0%, 1 = 1–25%, 2 = 25–50%, 3 = 50–100%). The final scores were determined by multiplying the two scores.

Hematoxylin and eosin (H&E) staining, transmission electron microscopy, and Western blot analysis were performed on thyroid gland tissues of Pink1 KO and WT mice. Detailed methods are provided in the Supplementary Materials and Methods.

5.8. Cell culture and transfection

HTori-3, a normal human thyroid follicular cell line, and Jurkat, a human T-lymphocyte cell line were used. Detailed information on cell culture and treatment are provided in the Supplementary Materials and Methods.

HTori-3 cells were transfected with siRNA for PINK1 (sc44598; Santa Cruz Biotechnology) and a nonspecific siRNA (sc37007; Santa Cruz Biotechnology) as a control using a transfection agent (lipofectamine RNAi MAX; Thermo Fisher Scientific). All transfections were performed in reduced-serum media (Opti-MEM™, Thermo Fisher Scientific) for 6 h, after which the medium was changed to RPMI-1640 (Welgene) with 10% FBS (Thermo Fisher Scientific) and 1% penicillin with streptomycin (Thermo Fisher Scientific) for the remainder of the experiment. The cells and supernatants were collected and processed for immunoblotting, RT-PCR, cell proliferation assay, and ELISA.

5.9. Cell proliferation assay

Cell proliferation assays were performed using the MTT assay (BIOSESANG, Sungnam, South Korea), according to the manufacturer’s protocol. Briefly, HTori-3 cells were seeded into 96-well plates at a density of 1×10^4 cells/well. After an incubation period of 24 h, the cells were treated with Mdivi-1 (5 µM) and cetuximab (50 µg/ml) after transfection, AREG (20 ng/ml), or transfection cell supernatant at 24 h and were transfected with siRNA for PINK1. Each well was treated with MTT solution (final concentration 0.5 mg/ml) and incubated for 4 h at 37°C. After the removal of the MTT solution, 200 µL of dimethyl sulfoxide was added to each well to dissolve the cells. All experiments were performed in triplicate, and the data are reported as cell proliferation at a wavelength of 570 nm.

5.10. RNA isolation and RT-PCR
Total RNA was isolated from siRNA-and siPINK1-transfected cells and cells treated with Mdivi-1 for 24 h using NucleoZOL following the manufacturer’s instructions. Briefly, RNA was reverse-transcribed into cDNA using Maxime™ RT PreMix (iNtRON Biotechnology, Sungnam, South Korea). mRNA levels were assessed by RT-qPCR using FastStart Essential DNA Green Master (Roche, Basel, Switzerland). All primers were purchased from BIONEER (Seoul, South Korea). The qPCR conditions were as follows: 10 min at 95°C, 45 cycles of 10 s at 95°C, 10 s at 60°C, 10 s at 72°C, melting at 0.2°C. RT-qPCR relative mRNA levels were normalized to the control and calculated using the comparative CT (2 − ΔΔCT) method, using GAPDH as a reference. Primer sequences are described in Supplementary Materials and Methods.

5.11. ELISA

Mouse serum thyroid-stimulating hormone levels were tested using a thyroid-stimulating hormone ELISA kit (MPTMAG-49K; EMD Millipore Corporation, Billerica, MA, USA) and measured using a Luminex 200 system (Thermo Fisher Scientific, Waltham, MA, USA). Moreover, IL-1β (DLB50), IL-6 (D6050), TNFα (DY225), and AREG (DAR00) protein concentrations were determined in supernatants of HTori-3 cells using commercial human ELISA kits (R&D Systems) according to the manufacturer’s instructions. The samples were analyzed in triplicate.

5.12. Mitochondrial ROS measurement

MitoSOX Red and H2DCFDA Green staining were performed to quantify mitochondrial or cellular ROS production in HTori-3 cells. We transferred 24-h cultured siPINK1-treated HTori-3 and control HTori-3 cells to MitoSOX and H2DCFDA solutions (5 µM). Stained HTori-3 cells were washed three times with M9 and analyzed using a 570-nm excitation fluorescence microscope.

5.13. Transwell migration assay

For the Jurkat cell migration assay, we collected the conditioned medium of siPINK1-transfected HTori-3 cells overnight and placed it in the lower chamber as a Jurkat cell chemoattractant. Jurkat cells were seeded into Matrigel: RPMI⁻/⁻ (dilution ratio, 1:15; Corning, Corning, NY, USA)-coated upper transwell chambers (pore size, 8 µm; Corning) and were incubated at 37°C. After 24 h of incubation, the non-migratory cells were removed using a cotton bud. The cells located on the transwell membrane were quantified using a cell counter (TC-20, BIO-RAD, San Francisco, CA, USA).

5.14. Statistical analyses

Statistical analyses were conducted using two-sided Student’s t-test. Box plot or violin with box plot shows the interquartile (IQR) range, whiskers indicate 1.5 × IQR. The dashed line in the center of the plot indicates the median value of all samples. Bar plots are presented as the mean ± SEM of multiple independent experiments. R version 4.2.1 (RRID:SCR_000432) was used for all statistical analyses. Statistical significance was defined as 2-sided p values < 0.05.

Declarations
Acknowledgments

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Author contributions

H.S.L., S-Y.L. and Y.S.S. designed this study. J.L., H-J. A., M-J. S., J-H. H. performed the experiments. H.S.L., J.L., and Y.S.S. collected and analyzed the data. H.S.L. and Y.S.S. contributed to manuscript preparation. All authors proofread and approved the manuscript.

Competing interests

All the authors declare no competing interests.

Conflict of interest

All the authors have no conflict of interest to disclose.

Availability of data and materials

The raw reads of RNA-sequencing analyses from thyroid glands of Pink1 knock-out/wild-type mice have been made publicly available in the NCBI GEO database (Accession: GSE232119, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE232119)

References


Figures
Figure 1

Downregulation of PINK1 was associated with lymphocytic thyroiditis (LT) and immune-related genes in human thyroids.

(A) PINK1 mRNA expression in human thyroids without LT and those with LT (n = 653; No LT = 589, LT = 64). mRNA expression value is normalized by variance stabilized transform (vst) function of DESeq2 R
package. A horizontal dashed line represents median value across all samples. (B) Pathway enrichment analysis of DEGs between \textit{PINK1}-high and \textit{PINK1}-low groups, which was determined based on the median expression of \textit{PINK1}, in human thyroids. The number inside the circles represent the count of DEGs belonging to the pathway. All top five enriched KEGG and GO terms were related to immune response. (C) Correlations between \textit{PINK1} mRNA expression and immune cell infiltration scores obtained using CIBERSORTx. Gray dots and pink dots represent thyroids without/with LT, respectively. (R = Pearson’s rho; Displays only cell types satisfied the criteria of $p < 0.05$ and $R \geq 0.2$). (D and E) Representative IHC images of PINK1 (D) and 8-OHdG (E) in human thyroids without/with LT. Bar plots represent the quantified IHC scores calculated by assessing the intensity and extent of the IHC ($n = 7$/each). Data in box plots are presented as mean $\pm$ SEM.
Figure 2

Mitophagy defects induced by Pink1-loss led to thyroid hyperplasia and inflammation in mouse thyroids (A) Gross images of Pink1WT and Pink1 KO mouse thyroid glands. Pink1 KO mice's thyroid glands were enlarged and represented goitrous nodular change as compared to Pink1 WT mice. (n =5/each) (B) Bar plot of thyroid gland weight of Pink1 WT/KO mice (n = 5/each). (C) Bar plot of serum thyroid-stimulating
hormone (TSH) level in Pink1WT/KO mice. (D) H&E staining images of thyroid glands of Pink1 WT (top; left, 400×; right, 1000×) and KO mice (bottom; left, 400×; right, 2000×). The red, gray, and pink boxes represent hyperplastic cell growth, enlarged follicles, and lymphocyte infiltration with a germinal center formation indicated by white, gray, and black arrows, respectively (the bottom right side of D). (E) Electron microscopic images of thyroid follicle cells of Pink1 WT (top; left, 20000×; right, 40000×) and KO mice (bottom; left, 12000×; right, 30000×). Mitochondria are marked with white arrows in Pink1 WT mouse thyroids, and black arrows in Pink1 KO mouse thyroids. Scale bars are as indicated. Data in bar plots are presented as mean ± SEM.
Figure 3

Mitophagy defects up-regulated damage-associated molecular pattern (DAMP) signaling, mtROS, and cytokine production in the thyroid

(A) Pathway enrichment analysis of DEGs between Pink1 KO and Pink1 WT mouse thyroids. All top five enriched KEGG pathways and GOBP/GOCC terms were related to immune response. (B and C)
Comparison of absolute scores of total immune cell abundance that calculated by CIBERSORTx (B) and ROS response gene set scores (C) between Pink1 WT and KO mouse thyroids (n = 5/each). (D) Box plots showing comparison of gene set scores of mitochondrial DAMP-related pathways between Pink1 WT and KO mouse thyroids (n = 5/each), along with heatmaps displaying the expression patterns of genes in each gene set. The gene sets include NLRP3 inflammasome, TLR9, STING, and NFkB pathways, which were derived from MsigDB. Data in box plots are shown in median and inter-quartile ranges, and horizontal dashed lines represent median value across the all samples. (E) Immunofluorescence staining of mtROS in human thyroid cells (HTori-3) transfected with control-siRNA (siCtrl) or PINK1-siRNA (siPINK1). Bar plots show relative intensity of each fluorescence of H2DCFDA and MitoSOX (n = 3/each). (F) Pro-inflammatory cytokine concentrations in the conditioned medium of HTori-3 cells after transfection with siCtrl or siPINK1 and after treatment with 0.5% DMSO (Ctrl) or 5μM of Mdivi-1, which were measured using ELISA. Data in bar plots are presented as mean ± SEM.
Figure 4

Mitophagy defects promoted cell proliferation by AREG secretion and activating EGFR pathway in the thyroid

(A) Representative AREG IHC images of human thyroids without/with LT (left, 400×; right, 1000×). Bar plot represents IHC scores of AREG in human thyroids without/with LT (n = 8/each). (B) AREG mRNA
expression of HTori-3 cells transfected with control-siRNA (siCtrl) or PINK1-siRNA (siPINK1), and treated with control (Ctrl) or Mdivi-1 (n = 6/each, normalized by β-actin expression). (cC AREG protein concentration in the conditioned medium of HTori-3 cells after transfection with siCtrl or siPINK1 and after treatment with Ctrl or Mdivi-1 (n = 6/each). (D) Box plots showing comparison of gene set scores of EGFR-related pathways between Pink1WT and KO mouse thyroids (n = 5/each), along with heatmaps displaying the expression patterns of genes in each gene set. The gene sets include EGFR, PI3K/AKT/mTOR, RAS/RAF/MEK/ERK, and cell cycle pathways, which were derived from MsigDB. Data in box plots are shown in median and inter-quartile ranges, and horizontal dashed lines represent median value across all samples. (E) Comparisons of proliferation rates using MTT assay between HTori-3 cells with siCtrl and siPINK1 (n = 6/each). (F) MTT assay on HTori-3 cells with siCtrl alone or with Cetuximab and siPINK1 alone or with Cetuximab (n = 5/each). Data in bar plots are presented as mean ± SEM.

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