mir-331 negatively regulates thyroglobulin secretion via ERp29

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Abstract: We investigated the effect of endoplasmic reticulum (ER) protein 29 (ERp29) on thyroglobulin (Tg) secretion and its negative regulation by microRNAs (miRNAs). ERp29 overexpression promoted Tg secretion from thyrocytes of PCCL3 cells via activation of ER stress sensors, including activation of transcription factor 6 fragmentation, XBP1 mRNA splicing by inositol-requiring enzyme 1, and phosphorylation of eukaryotic initiation factor 2 alpha, as downstream actions of RNA-dependent protein kinase-like ER kinase. Thyroid hormone receptor beta is a target gene of mir-331, one of the most abundant miRNAs observed in ERp29-overexpressing cells. mir-331 negatively regulated Tg expression and secretion by ~70% compared with the control. To overcome hypothyroidism, Tg secretion at the molecular level is required to counteract negative regulation of intracellular mir-331. Our findings may provide insight into the treatment of diseases caused by poor ER protein secretion, including ER storage diseases (ERSDs).

Key words: PCCL3 cells, endoplasmic reticulum (ER) stress, ERp29, thyroglobulin (Tg), mir-331

The major biological function of the endoplasmic reticulum (ER), an intracellular organelle found in all eukaryotic cells, is post-translational modification of secretory proteins (Braakman and Bulleid, 2011). The ER has a complex signal-transducing system that senses and responds to pathophysiological changes to maintain cellular homeostasis (Ron and Harding, 2012). During adaption and survival of cells and/or tissues, ER stress is induced by the unfolded protein response (UPR) via expression of chaperones in the ER, including binding immunoglobulin protein (BiP), glucose-regulated protein 94, calnexin, protein disulfide isomerase (PDI), and ER protein 29 (ERp29) (Adams et al., 2019). Such factors directly or indirectly mediate multiple molecular biological processes via three kinds of ER stress sensors: inositol-requiring enzyme 1 (IRE1), protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Back and Kaufman, 2012). The ER stress response in mammalian cells is triggered by the dissociation of BiP from transducers such as PERK, ATF6, and IRE1. BiP binds to unfolded proteins present in the ER lumen and activates the ER stress response (Frakes and Dillin, 2017; Gardner et al., 2013).

Several types of ER chaperones are involved in nascent polypeptide folding and maturation, including ERp29, the full-length cDNA (encoding a 29 kDa protein) of which was first isolated in 1997 (Demmer, 1997). ERp29 is expressed ubiquitously and abundantly and is localized mainly in the ER lumen. It belongs to the PDI protein family, although it lacks an active thioredoxin motif, suggesting that this protein might not be a disulfide isomerase (Rainey-Barger et al., 2007). ERp29 may have a variety of functions, as its expression is induced by radiation and thyroid-stimulating hormone (TSH), and its high expression in several cancers and in dental enamel cells (Frakes and Dillin, 2017; Kwon et al. 2000). Although ERp29 deficiency does not alter thyroglobulin (Tg) expression levels (Park et al., 2005), ERp29 has been shown to play an important role in the folding and assembly of secretory proteins of Tg (Baryshev et al., 2006) in FRTL-5 Fisher rat thyrocytes. Further studies on ERp29 function are required to determine the most important biological functions of ERp29. This study investigated the effect of ERp29 on Tg secretion and its negative regulation by microRNAs (miRNAs) to provide a better understanding of ERp29 function.

The rat thyroid cell line PCCL3 (RRID:CVCL_6712) was cultured in Coon's medium in a humidified atmosphere containing 5% CO_2 (Park et al., 2005). For each individual experiment, the PCCL3 was washed three times with cold phosphate-buffered saline to completely remove the hormones and then incubated in growth medium without hormones for 48 h. PCCL3 cells were scraped, lysed, and separated by adding sodium dodecyl sulfate sample buffer, followed by polyacrylamide gel electrophoresis (SDS–PAGE). Each samples (10 μg / μL) was loaded into each well of the gel. The processing of Western blotting has previously been described (Park et al., 2005). Rabbit anti-eukaryotic initiation factor (eIF) 2

antibody, phosphorylated eIF2 antibody, and goat anti-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tg secretion in cell medium was analyzed by Western blotting using an anti-Tg antibody.

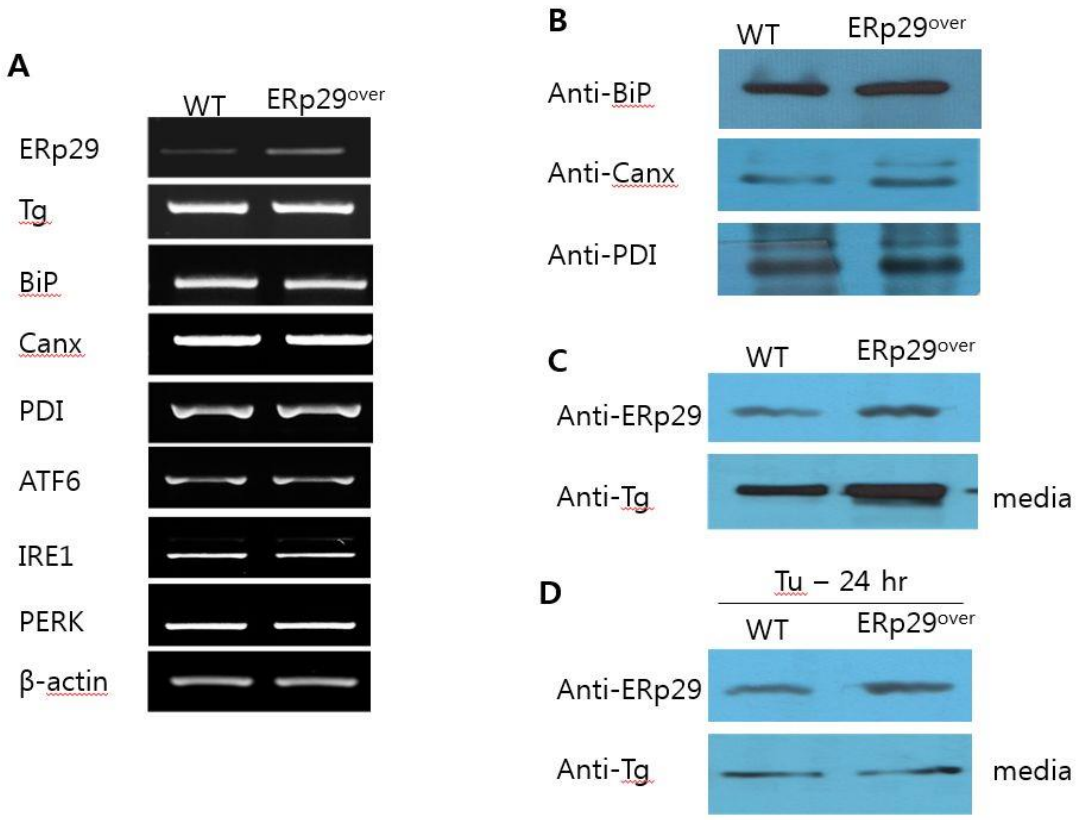
Reverse-transcription polymerase chain reaction (RT-PCR) was performed with total RNA using forward and reverse primers (Bioneer Co., Daejeon, Korea) specific to IRE1, 5′-ACCACCAGTCCATCGCCATT-3′ and 5′-CCACCCTGGACGGAAGTTTG-3′; BiP, 5′-AGTGGTGGCCACTAATGGAG-3′ and 5′-TCTTTTGTCAGGGGTCGTTC-3′; ATF6, 5′-CTAGGCCTGGAGGCCAGGTT-3′ and 5′-ACCCTGGAGTATGCGGGTTT-3′; PDI, 5′-ATCGAGTTCACCGAGCAGAC-3′ and 5′-TCACAGCTTTCTGGTCATCG-3′; PERK, 5′-GGTCTGGTTCCTTGGTTTCA-3′ and 5′-TTCGCTGGCTGTGTAACTTG-3′; GAPDH, 5′-ACATCAAATGGGGTGATGCT-3′ and 5′-AGGAGACAACCTGGTCCTCA-3′; and X-box DNA-binding protein 1 (XBP1), 5′-AAACAGAGTAGCAGCTCAGACTGC-3′ and 5′-TCCTTCTGGGTAGACCTCTGGGAG-3′, respectively. RT-PCR conditions have previously been described (Kwon et al., 2007). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). XBP1 splicing: Total RNA was reverse-transcribed into cDNA. Double-stranded cDNA was synthesized by PCR using sense (5′-AAACAGAGTAGCAGCGCAGACTGC-3′) and anti-sense (5′-TCCTTCTGGGTAGACCTCTGGGAG-3′) primers specific to XBP1. Amplified cDNA was treated with *Pst*I. The resulting product was analyzed by electrophoresis using a 2% agarose gel. RNA interference: Rat ERp29 small interfering RNA (siRNA) and mir-331 were obtained from Bioneer Co. (Daejeon, Korea). Transfection was conducted using Lipofectin reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Briefly, siRNA (20 μM) was diluted in 100 μL Opti-MEM I reduced serum medium (Invitrogen) and mixed with 3 μL of each transfection reagent (Invitrogen) pre-diluted in 97 μL Opti-MEM. PCCL3 cells were transfected with siRNA using Opti-MEM for 24 h. PCCL3 cells transfected with a non-target control siRNA were used as controls. The pcDNA $3.1^{(+)}$ vector (Invitrogen) was used for overexpression experiments.

Tg, a large soluble protein with a molecular weight of 660 kDa, is present in the colloid of the thyroid follicle, where iodine is synthesized into two types of thyroid hormones: triiodothyronine (T3) and tetraiodothyronine (T4). T3 and T4 are critical for development, growth, and metabolism, exerting the most prominent effects during fetal development and early childhood (Citterio et al., 2019). Deficiency in the production or activity of thyroid hormones can lead to hypothyroidism, one of the most frequent hormone diseases (Mendoza and Hollenberg, 2017). Hypothyroid individuals exhibit various physiological changes, including impaired endothelial function, left ventricular systolic and diastolic dysfunction, and dyslipidemia with elevated total cholesterol and low-density lipoprotein-cholesterol

levels and a decreased high-density lipoprotein-cholesterol level (Schübel, 2017). Numerous molecular studies have been conducted on hypothyroidism. We previously reported that congenital hypothyroidism is caused by an ER storage abnormality due to a single amino acid substitute in the acetylcholinesterase-like domain of Tg (Kim et al., 1996). Growth, proliferation, and Tg secretion from FRTL-5 cells are dependent on TSH (Kim et al., 1998). We showed that ERp29 promotes Tg secretion induced by TSH to overcome hypothyroidism (Park et al., 2015). ERp29 interacts with BiP under ER stress and is considered a crucial player in the ER stress response.

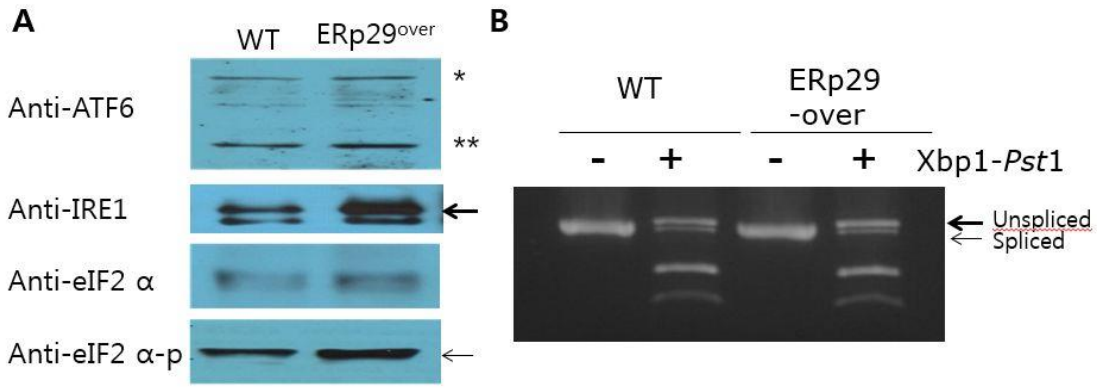
This study investigated the mechanism of Tg secretion induced by ERp29 overexpression on the gene expression of ER chaperones and ER stress sensors in PCCL3 cells. Although the expression of ERp29 was elevated by ERp29 overexpression, transcript levels of other genes, including Tg, were unchanged (Figure 1A). However, translation levels of ER chaperones were upregulated by ERp29 overexpression (Figure 1B). These results suggest that ERp29 is associated with the regulation of ER chaperones in the ER lumen at the protein biosynthesis rather than transcription level. Next, we investigated the effect of ERp29 overexpression on Tg secretion by PCCL3 cells. Interestingly, Tg secretion was promoted by ERp29 overexpression (Figure 1C). Although ERp29 expression was involved in promoting Tg secretion (Figure 1C), ER stress treated by tunicamycin inhibited extracellular secretion of Tg (Figure 1D). These results suggest that ERp29 participates as an ER chaperone in ER quality control (ERQC), potentially to aid secretary protein folding in the ER lumen, but not gene transcription control.

Figure 1.



We investigated the effect of ERp29 overexpression on the regulation of ER stress sensors. Three distinct ER stress sensors (IRE1, PERK, and ATF6) participate in the UPR in mammalian cells. These ER stress sensors are downstream effectors of ER chaperone activity, and transmit signals from the ER to the nucleus in response to chaotic protein fold processing in the ER lumen. Activation (autophosphorylation and dimerization) of IRE1 promotes the endonuclease domains, which cleave XBP1 mRNA, generating an activated form of XBP1. PERK activation also results in phosphorylation of a subunit of eIF-2, thereby inhibiting translation initiation. ATF6 is cleaved at the cytosolic face of the membrane in response to ER stress, and its N-terminal cytoplasmic domain, which contains DNA-binding, dimerization, and transactivation domains, is translocated to the nucleus. Subsequently, binding to both the ER stress response elements and ATF6-binding sites of ER chaperone genes enhances their expression (Back and Kaufman, 2012). As shown in Figure 2A, ERp29 overexpression promotes cleavage of the N-terminus of ATF6, upregulation of IRE1, and phosphorylation of eIF2 alpha. In addition, ERp29 overexpression promoted XBP1 mRNA splicing downstream of IRE1 (Figure 2B). These results demonstrate that ERp29 overexpression activated an ER chaperone, which in turn was transmitted to activate ER stress sensors located on the ER membrane.

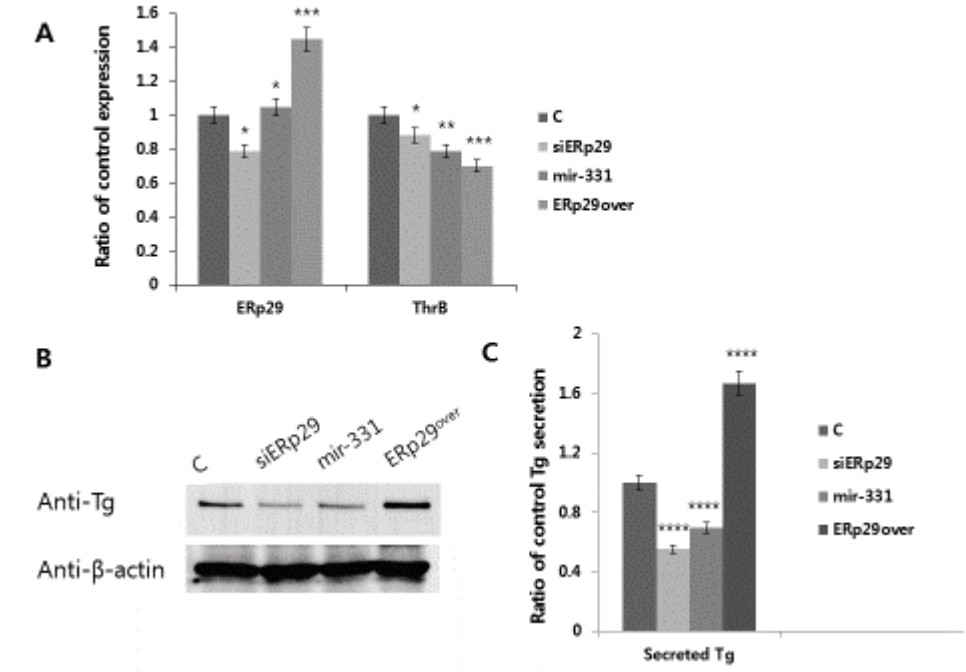
Figure 2.



In this study, miRNA and siRNA were used to inhibit the expression of ERp29. miRNAs, small non-coding RNAs consisting of approximately 22 nucleotides, function in post-transcriptional regulation of gene expression, including RNA silencing (Liu et al., 2017). Although they exhibit only partial complementation with their target mRNAs, and cannot cleave mRNAs, they inhibit the conversion of mRNA into proteins. siRNA, a double-stranded RNA consisting of 20–25 base pairs, is the most common RNA interference method for silencing specific genes. We examined whether an siRNA targeting ERp29 (siERp29) and miRNA-331 (mir-331) modulates ERp29 expression in PCCL3 cells. mir-331 was among the most abundant miRNAs detected in PCCL3 cells overexpressing ERp29. One target gene of mir-331 is thyroid hormone receptor beta (ThrB) (GenBank accession no.: NM 001270854)

(Jazdzewski, 2011). ERp29 expression was downregulated in cells treated with siERp29 (Figure 3A) whereas no significant difference in expression was detected following miRNA-331 treatment (Figure 3A, left). However, mir-331 treatment reduced ThrB expression by about 20%, and ERp29 overexpression decreased ThrB expression by about 30% (Figure 3A, right). These results suggest that siRNA-mediated knockdown inhibits the expression of ERp29 mRNA, and that the expression of ERp29 and Tg are functionally similar. We also assessed the effects of siERp29 and mir-331 on Tg expression under the same experimental conditions (Figure 3B). siERp29 treatment strongly reduced Tg expression, to a slightly greater extent than mir-331. Tg secretion levels showed nearly the same pattern as Tg expression (Figure 3C). When ERp29 overexpression used as a control, Tg secretion increased by about 1.7-fold, whereas Tg secretion decreased in cells treated with siERp29 and mir-331, by 40% and 30%, respectively. These results suggest that Tg secretion from PCCL3 cells is positively related to ERp29 gene expression, whereas mir-331 negatively regulates Tg expression and secretion through ThrB expression regulation.

Fig.3.



In summary, ERp29 overexpression upregulated Tg expression during translation (including Tg secretion in PCCL3 cells) via ER stress signal sensors: ATF6 fragmentation, XBP1 mRNA splicing by IRE1 activation, and eIF2 alpha phosphorylation. The most significant result of this study is that mir-331 inhibited both Tg expression and secretion compared with the control. Thus, to overcome hyperthyroidism, further studies of the regulation of ERp29 associated with mir-331 are required.

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Conflict of interest Authors state no conflict of interest.

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Figure legends

Figure 1. Gene expression of endoplasmic reticulum (ER) chaperones and Tg secretion by ERp29 overexpression. (A) In PCCL3 and ERp29^{over} PCCL3 cells, gene expression was estimated for both ER chaperones and ER stress sensors using reverse-transcription polymerase chain reaction (RT-PCR). (B) ER chaperone protein expression was measured using Western blotting. (C) Tg secretion by ER29 was measured using Western blotting. (D) Tg secretion was measured using tunicamycin treatment followed by Western blotting. All experimental conditions are described in detail in the Materials and Methods section. Data represent means of at least three independent experiments.

Figure 2. Regulation of ER stress sensors by ERp29 overexpression. (A) Results of Western blotting in PCCL3 and ERp29^{over} PCCL3 cells, showing full-length ATF6 (*), partial-length ATF6 (**), IRE1 (←), and phosphorylated eIF2 alpha (←). (B) RT-PCR analysis showed the expression of two isoforms: spliced (XBP1S) and un-spliced (XBP1U) xbp1 transcripts. All experimental conditions are described in detail in the Materials and Methods section. Data represent means of at least three independent experiments.

Figure 3. Regulation of Tg expression and secretion by small interfering RNAs. (A) Regulation of ERp29 and ThrB expression using siERp29 and miRNA-331, respectively. (B) Regulation of Tg gene expression by siERp29 and miRNA-331. (C) Regulation of Tg secretion by siERp29 and miRNA-331. All experimental conditions in Figure 1 are described in the Materials and Methods section. Data represent means ± standard deviation (SD) of at least three independent experiments. Statistical significance between multiple groups: one-way analysis of variance (ANOVA) test. GraphPad Prism 6 software (GraphPad Software Inc.). *$P < 0.05$ **$P < 0.005$, ***$P < 0.001$, ****$P < 0.0001$.