


ORIGINAL ARTICLE OPEN ACCESS

Melatonin Induces PERK-ATF4 Unfolded Protein Response and Apoptosis in Human Choriocarcinoma Cells

Josianne Bienvenue-Pariseault^{1,2} | Lucas Sagrillo-Fagundes^{1,2} | Philippe Wong-Yen¹ | Darius Stakamatos¹ | Marie Cohen³ | Cathy Vaillancourt^{1,2} 

¹Institut national de la recherche scientifique (INRS) - Centre Armand-Frappier Santé Biotechnologie (AFSB), Laval, Canada | ²CIUSSS-Nord-de-l'île-de-Montréal, Montreal, Canada | ³Department of Pediatrics, Gynecology and Obstetrics, Faculty of Medicine and Translational Research Centre in Onco-hematology, University of Geneva, Geneva, Switzerland

Correspondence: Cathy Vaillancourt (cathy.vaillancourt@inrs.ca)

Received: 13 January 2025 | **Revised:** 8 August 2025 | **Accepted:** 19 August 2025

Funding: This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) (06778-2019) to C.V.

Keywords: apoptosis | BeWo cell | choriocarcinoma | indolamine | melatonin | PERK pathway | unfolded protein response

ABSTRACT

Melatonin, an indolamine primarily recognized for regulating circadian rhythms, has also demonstrated notable antitumoral properties. Melatonin induces endoplasmic reticulum (ER) stress, modulates autophagy, and promotes apoptosis in various tumors, including gastric, ovarian, cervical, oral tongue, colorectal, renal, hepatic, and bladder cancer. In placental choriocarcinoma, melatonin reduces cell viability and induces apoptosis by inhibiting autophagy and disrupting the mitochondrial membrane potential. However, its effects on ER stress and the unfolded protein response (UPR) pathway remain unexplored. It is hypothesized here that the proapoptotic effects of melatonin in choriocarcinoma cells occur through the activation of the UPR pathway. The factors implicated in the UPR (PERK, IRE1 α , ATF6, GRP78, ATF4, CHOP, P-eIF2 α) pathways were evaluated by Western blot, RT-qPCR, and flow cytometry in BeWo (human choriocarcinoma) cells treated with or without melatonin (1 mM). Melatonin significantly increased protein levels of GRP78 ($p = 0.0329$), IRE1 α ($p = 0.0394$), p-eIF2 α ($p = 0.0439$), ATF4 ($p = 0.0267$), CHOP ($p = 0.0379$), Bax and cleaved PARP but did not affect TRAF2 and NF κ B protein levels nor XBP1 mRNA splicing. PERK knockdown, via siRNA, prevented the rise in GRP78, p-eIF2 α /eIF2 α , and ATF4 levels by melatonin. Additionally, melatonin increased early apoptosis in BeWo cells ($p = 0.0371$) and PERK knockdown increased the susceptibility of BeWo cells to apoptosis when treated with tunicamycin ($p = 0.0359$), suggesting that ER stress plays a role in BeWo cell survival. This study demonstrates that melatonin activates the PERK-ATF4-P-eIF2 α -CHOP pathway and induces early apoptosis in BeWo cells, while PERK deficiency compromises cell survival under ER stress. Our findings suggest that modulating PERK-UPR signaling with melatonin could present a promising therapeutic strategy for cancer, including placental choriocarcinoma.

1 | Introduction

The endoplasmic reticulum (ER) is a multitask organelle that assures Ca²⁺ storage/distribution, lipid biosynthesis, translation, posttranslational modifications and folding of proteins [1]. Physiological stressors, such as nutrient deprivation [2], acidosis [3], and

oxidative stress [4], can compromise ER functions, thereby inducing ER stress. Altered ER function can lead to an accumulation of unfolded or misfolded proteins that trigger the unfolded protein response (UPR). The UPR can restore cell homeostasis by expressing chaperones that help in folding proteins. This process eliminates unfolded proteins through autophagy and proteasomal

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2025 The Author(s). *Journal of Pineal Research* published by John Wiley & Sons Ltd.

degradation, primarily via the activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring transmembrane kinase/endonuclease 1 α (IRE1 α) and activating transcription factor 6 (ATF6) [5–7]. When the ER stress is prolonged, the UPR pathway can no longer restore cellular homeostasis, shifting instead to a proapoptotic response and away from an adaptive response [8, 9].

The UPR pathway has significant roles in tumorigenesis, metastasis, and survival. PERK, IRE1 α and ATF6 are crucial for the adaptation of cancer cells to their changing environment [10]. Anticancer drugs, with reactive oxygen species (ROS)-modulating ability, have been shown to counteract cancer cell adaptation by inducing ROS generation causing excessive ER stress, thereby promoting apoptosis in cancer cells [11]. For example, many studies showed that *N*-acetyl-5-methoxytryptamine (melatonin) induces apoptosis through the UPR pathway activation in gastric [12], bladder [13], hepatic [14, 15], skin [16], tongue squamous carcinoma [17], and colorectal human cancer [18].

Melatonin is widely known as pineal gland derived circadian regulator [19]. However, this amphiphilic indolamine is also synthesized by all organs and tissues, including placental trophoblast cells [20, 21]. Melatonin can cross easily through lipophilic barriers and is known to have anti-inflammatory [22], antioxidants [23], antiapoptotic [24], and antitumoral functions [25] in healthy cells subjected to stressful conditions. Paradoxically, melatonin has been shown to inhibit cell proliferation [26], migration [27], angiogenesis [28], and metastasis [25], as well as inducing metabolic dysregulation [29], promoting apoptosis [30], and triggering pro-inflammatory [31] responses across diverse tumor cell types [32, 33]. The use of melatonin as a therapeutic agent in cancer is promising due to its low toxicity, ease of synthesis and its ability to protect healthy cells submitted to stress, including chemotherapy side-effects [34–37]. Indeed, we have previously shown that melatonin induces choriocarcinoma cell death by inhibiting autophagy, decreasing cell viability and disrupting mitochondrial membrane potential whilst, in contrast, being protective in normal primary human villous trophoblast submitted to hypoxia/reoxygenation (H/R) by inducing autophagy, reducing oxidative stress and inflammation [35, 38–40]. However, the effect of melatonin on ER stress in choriocarcinoma cells has never been studied. In tongue squamous carcinoma [17], colorectal carcinoma [41], lung [42], liver [15, 43] and cervical cancer [44], induction of oxidative stress and autophagy by melatonin was associated with the induction of ER stress [33].

Choriocarcinoma is a rare and highly aggressive neoplasm that originates from the malignant transformation of trophoblasts [45]. Even though most choriocarcinoma patients are successfully treated with a single-agent chemotherapy, others die despite multi-drug resistance [46]. Therefore, novel therapy strategies are needed to improve the prognosis of this group of patients [46]. Utilizing melatonin to disrupt UPR signaling and alter the delicate equilibrium between adaptive UPR and UPR-induced apoptosis could present an innovative therapeutic strategy [47, 48].

Overall, we propose that melatonin induces UPR pathways in choriocarcinoma cells. Therefore, the present study aimed to

investigate melatonin antitumoral effects through the modulation of UPR in BeWo choriocarcinoma cells.

2 | Materials and Methods

2.1 | BeWo Cell Line Culture

BeWo cells (#CCL-98; ATCC, MD, US) were cultured in T-75 flask cm² (Corning Life Science, Arizona, US) at 37°C and 5% CO₂, as previously described [38]. The culture medium used was Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F12) without phenol red, supplemented with 0.6 g/L sodium bicarbonate (NaHCO₃) (Sigma-Aldrich, ON, Canada) and 10% fetal bovine serum (FBS; Wisent, QC, Canada), and was refreshed every 2 days. Subculturing was performed when cells reached 90%–95% of confluence.

2.2 | Cell Treatments

Cells between passages 6 and 18 were seeded at a density of 2.5 × 10⁵ cells/mL in 6-well plate and cultured under physiological normoxia (8% O₂, 5% CO₂, 87% N₂) for 24-h, in a Modular Incubator Chambers (Billups-Rothenberg, CA, USA), to ensure cell adherence, as we previously described [49]. Subsequently, the cells were treated with melatonin (Mel, 1 mM) (Sigma-Aldrich) or with the vehicle control dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 0.1% (final concentration) or with 3.5 μg/mL of tunicamycin (Tm, ER stress positive control; Cayman chemicals, MI, US) and maintained under normoxia for 24-h. Melatonin solution diluted in 0.1% DMSO was freshly prepared before each experiment [38]. A dose–response curve was conducted using increasing melatonin concentrations (1 nM, 1 μM, and 1 mM) to determine the concentration with the maximal effect, with melatonin (1 mM) being selected (Figure S1).

2.3 | Protein Expression Analysis

Protein expression of BeWo cells treated with or without melatonin was analyzed by immunoblot [22, 38, 50]. Proteins were extracted using RIPA buffer (50 mM TRIS-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, NP-40 (Nonidet P-40) 1%, Na-deoxycholate 0.25%) supplemented with protease (Invitrogen, ON, Canada) and phosphatase inhibitors (Roche, ON, Canada). The concentration in each sample was determined by spectrometry using the bicinchoninic acid (BCA) assay according to the manufacturer's instructions (Pierce, Biotechnology, CA, USA). Proteins (35 μg) were separated by electrophoresis on 8%, 12% or 10%–15% SDS-PAGE and transferred to nitrocellulose membranes using the Trans-Blot Turbo (BioRad, QC, Canada). To block nonspecific binding, the membrane was incubated in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA) or 5% nonfat dry milk. Primary antibodies were incubated overnight at 4°C (Table 1). Subsequently, incubation with the appropriate HRP-conjugated secondary antibodies was carried out for 1-h at room temperature (Table 1). Blots were developed with Clarity Western ECL substrate or Clarity Max Western ECL substrate (BioRad) and the chemiluminescence was detected with the ChemiDoc XRS+ system (BioRad). Band

TABLE 1 | List of antibodies used for Western blot analysis.

Antibody	Source	Molecular weight (kDa)	Dilution	Supplier
GRP78	Rabbit	78	1:1000	Cell signaling, #3177
IRE1 α	Rabbit	130	1:1000	Cell signaling, #3294
TRAF2	Rabbit	53	1:1000	Abcam, #ab126758
PERK	Rabbit	140	1:1000	Cell signaling, #5683
Phospho-eIF2 α	Rabbit	38	1:500	Abcam, #32157
eIF2 α	Rabbit	38	1:500	Cell signaling, #9722
ATF4	Rabbit	49	1:500	Cell signaling, #11815
CHOP	Rabbit	27	1:1000	Cell signaling, #2895
NF- κ B (p65)	Rabbit	65	1:1000	Cell signaling, #8242
I κ B	Rabbit	39	1:1000	Cell signaling, #4812
Bax	Rabbit	20	1:500	Cell signaling, #2774
Bcl-2	Mouse	26	1:500	Cell signaling, #15071
ATF6 α	Rabbit	74	1:500	Abcam, #ab227830
Cl-PARP	Rabbit	89	1:500	Cell Signaling, #5625
Anti-Mouse HRP	—	—	1:5000	Millipore (AP192P)
Anti-Rabbit HRP	—	—	1:5000	Millipore (AP182P)

Abbreviations: ATF4, activating transcription factor 4; Bax, Bcl-2-associated X; Bcl-2, B-cell lymphoma 2; CHOP, DNA damage-inducible transcript 3, also known as C/EBP homologous protein; eif2 α , eukaryotic initiation factor 2; GRP78, 78-kDa glucose-regulated protein; HRP, horseradish peroxidase; I κ B, inhibitor of nuclear factor kappa B; IRE α , serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α ; NF κ B, nuclear factor-kappa; P-eif2 α , phosphorylation of the eukaryotic initiation factor 2; TRAF2, TNF receptor-associated factor 2; XBP1, X-box binding protein 1.

TABLE 2 | Primers used for RT-qPCR analysis.

Primers	Primer sequences (5' -> 3')	Amplicon size (nt)	References
XBP1s	Foward: TGCTGAGTCCGCAGCAGGTG Reverse: GCTGGCAGGCTCTGGGGAAG	169	(von Loeffelholz et al., 2017)
XBP1 total	Foward: GTGAGCTGGAACAGCAAGTGGT Reverse: CCAAGCGCTGTCTTAACTCCTG	126	(von Loeffelholz et al., 2017)
B2M	Foward: GATGAGTATGCCTGCCGTGT Reverse: CTGCTTACATGTCTCGATCCCA	79	Primer Blast
SDHA	Foward: TACAAGGTGCGGATTGATG Reverse: CGATCACGGGTCTATATTCAA	148	Primer Blast

Abbreviations: B2M, beta-2-microglobulin; SDHA, succinate dehydrogenase complex flavoprotein subunit A; XBP1s, cleaved X-box binding protein; XBP1, X-box binding protein 1.

quantification was carried out by densitometric analysis, using Image Lab 5.2 software (BioRad). The normalization of the proteins was done with total protein using the MemCode Reversible Stain Kit, according to the manufacturer's instructions (Thermo Fisher Scientific, ON, Canada). Total protein normalization was used to avoid variability under stress conditions and ensure accurate quantification as previously described [50, 51]. Before this, we prepared a standard curve using serial dilutions of the protein samples to determine the optimal concentration of primary antibodies to use according to Taylor et al. recommendations [50, 51].

2.4 | mRNA Expression Analysis

BeWo cells treated or not with melatonin were analyzed by real time quantitative polymerase chain (RT-qPCR), according to

the MIQE guidelines [52, 53]. Total RNA was extracted with the RNeasy mini kit, according to the manufacturer's instructions (Qiagen, ON, Canada). RNA purity and integrity were respectively assessed by spectrometry with the Nanodrop and by electrophoresis of the RNA on agarose gel, respectively. The samples that satisfied the purity and the integrity were processed to cDNA using the iScript Reverse Transcription Supermix for RT-qPCR (BioRad). cDNA was then amplified with the SsoAdvanced Universal SYBR Green Supermix (BioRad) and specific primers (Table 2). Beforehand, amplification efficacy of the primers was determined by doing a standard curve and primer's annealing temperature was confirmed by doing a thermal gradient. Experiments were run on a CFX96 Real-Time PCR Detection System (BioRad). Gene expression was normalized to beta-2-microglobulin (B2M) and succinate dehydrogenase complex flavoprotein subunit A (SDHA) and expressed as a relative expression.

2.5 | Quantification of Apoptosis by Flow Cytometry

Annexin V-(Fluorescein) FITC/Propidium iodide (PI) kit (eBiosciences, ON, Canada) was used to analyze early and late apoptosis by flow cytometry. As a positive control, BeWo cells were treated with staurosporine (1 mM) (Sigma-Aldrich) for 24-h. Cells were harvested with TrypLE Express Enzyme 1× (Gibco, ON, Canada) and washed with PBS. Cells (2.5×10^5 cells/mL) were resuspended in binding buffer provided by the manufacturer. Annexin V-FITC and PI staining were done according to the manufacturer's instructions. Briefly, 5 μ L of Annexin V-FITC was added to 195 μ L of cell suspension and incubated for 10-min at room temperature. PI (20 μ g/mL) was added to one sample at a time, for 1-min at room temperature just before reading, to decrease the false positive risk. Quantification of apoptotic cells by flow cytometry was done using FACSCalibur (BD Biosciences, CA, US). Data were analyzed by using FlowJo software (BD Biosciences).

2.6 | siRNA Reverse Transfection

Human PERK-specific short interfering RNA (siRNA) or scrambled RNA (control) was mixed with serum-free Opti-MEM medium for 5-min before adding Lipofectamine RNAi-MAX for 20-min in a 24-well plate (Life Technologies, CA, USA). BeWo cells (17.5×10^4 cells/mL) were then added to the wells and cultured for 72-h under normoxic conditions. Subsequently, cells were treated with DMSO (vehicle control), melatonin, or tunicamycin for 24-h in normoxia. Human PERK-specific siRNA and scrambled RNA were purchased from Santa Cruz (TX, US).

2.7 | Statistical Analysis

All data represent at least four different BeWo cell passages. Statistically significant differences ($p < 0.05$) were assessed using GraphPad Prism 9 (version 9.5.1). Student *t*-test was used when two treatments groups were compared while one-way analysis of variance (ANOVA) followed by the Tukey post-hoc test was used when more than two treatment groups were compared.

3 | Results

3.1 | Melatonin Activated the Unfolded Protein Response (UPR) in BeWo Cells

To determine if melatonin activates the UPR in BeWo cells, we investigated the protein levels of the first UPR mediators, namely Glucose-Regulated Protein 78 (GRP78). Tunicamycin, used as positive control for UPR activation, increased GRP78 protein level compared to vehicle control. However, as a positive control, tunicamycin was not included in the statistical analysis. Melatonin (1 mM) increased GRP78 protein level by 3.2-fold in BeWo cells compared to the vehicle control (Figure 1). This suggests that melatonin activate the UPR in BeWo cells.

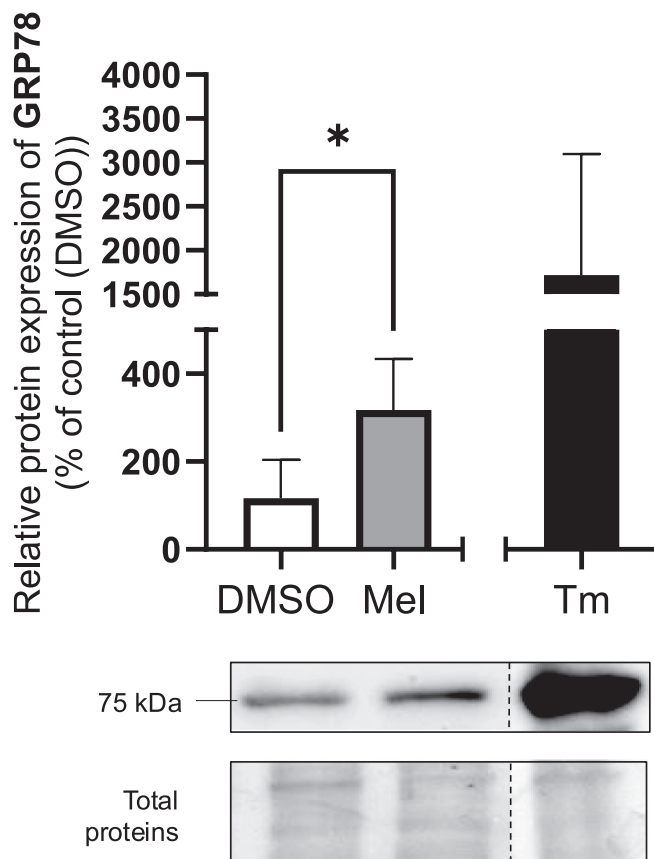


FIGURE 1 | Melatonin increases unfolded protein (UPR) response in BeWo cells. BeWo cells were treated with the vehicle control (0.1% DMSO) with or without melatonin (1 mM) or Tm (3.5 μ g/mL) under normoxia (8% O₂) conditions for 24-h. GRP78 protein level was determined by Western blot. Equal protein amounts of cell lysates (35 μ g) were subjected to Western blot assay using anti-GRP78. Total protein was used for normalization. GRP78: 78-kDa glucose-regulated protein; DMSO: dimethylsulfoxide; Mel: melatonin; Tm: tunicamycin; Data are shown as mean \pm SD and were analyzed using an unpaired *t*-test (DMSO vs Mel, * $p < 0.05$), $n = 5$. To facilitate readers' experiences, the certain band order has been changed. Those modifications have been identified with dotted lines. Original western blots are included under Figure S2.

3.2 | Melatonin Has No Effect on the ER Membrane Transducer IRE α and ATF6 Pathways

To investigate whether melatonin activates the IRE1 α and ATF6 arms of the UPR in BeWo cells, we assessed the expression of key proteins and genes involved in these pathways. Specifically, to evaluate the IRE1 α arm, we measured the protein levels of TNF receptor-associated factor 2 (TRAF2), Nuclear factor-kappa B (NF κ B), and inhibitor of nuclear factor kappa B (I κ B) by immunoblotting [54, 55]. In addition, we analyzed the gene expression of *XBPI* (X-box binding protein 1) and its spliced form, *sXBPI*, using RT-qPCR (Figure 2). As with tunicamycin, melatonin significantly increased IRE1 α protein level (5.1-fold) compared to the vehicle control (Figure 2A). However, TRAF2 protein levels remained unchanged following exposure to melatonin or tunicamycin, compared to the vehicle control (Figure 2B). In addition, melatonin had no effect on the NF κ B/I κ B or *XBPI*/*XBPIs* ratios, whereas tunicamycin

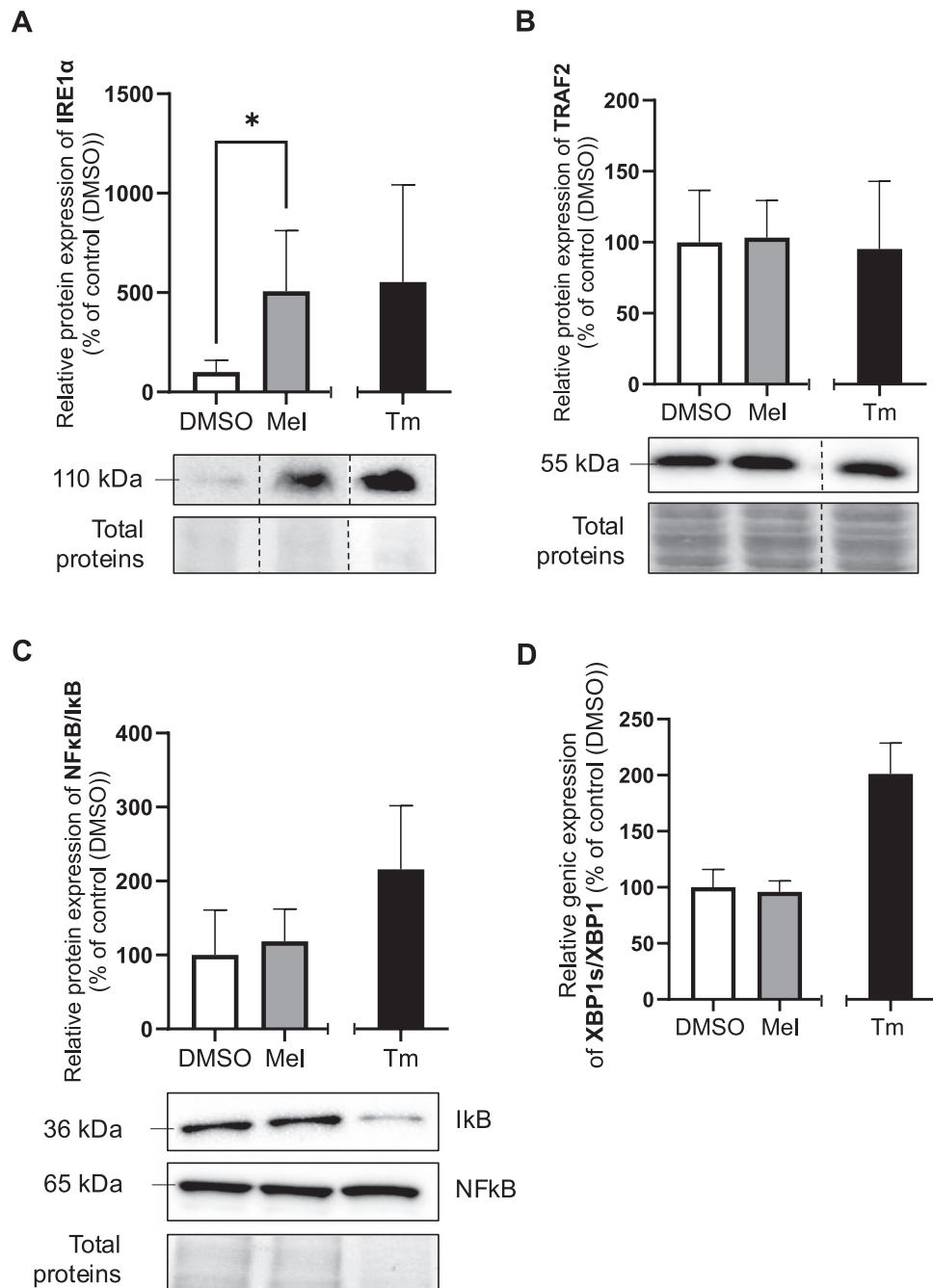


FIGURE 2 | Melatonin has no effect on the protein level of factors implicated in IRE α -UPR pathway. BeWo cells were treated with the vehicle (DMSO 0.1%) or melatonin (1 mM) or Tm (3.5 μ g/mL) under normoxia (8% O₂) conditions during 24-h. (A) IRE α , (B) TRAF2 and (C) I κ B/NF κ B ratio protein level was determined by Western blot. Equal protein amounts of cell lysates (35 μ g) were subjected to Western blot assay using anti-IRE α , anti-TRAF2, anti-NF κ B and anti-I κ B. Total protein was used for normalization. (D) XBP1s/XBP1 gene expression was determined by RT-qPCR using specific primers. *B2M* and *SDHA* were used as references genes for normalization. DMSO: dimethylsulfoxide; I κ B: inhibitor of nuclear factor kappa B; IRE α : serine/threonine-protein kinase/endoribonuclease inositol-requiring enzyme 1 α ; Mel: melatonin; NF κ B: nuclear factor-kappa B; TRAF2: TNF receptor-associated factor 2; Tm: Tunicamycin; XBP1: X-box binding protein 1; XBP1s: spliced X-box binding protein 1; Data are shown as mean \pm SD and were analyzed using an unpaired t-test (DMSO vs Mel, * p < 0.05), n = 4–5. To facilitate readers' experiences, the band order of certain blots has been changed. Those modifications have been identified with dotted lines. Original western blots are included under Figures S5–S8.

increased both (Figure 2C,D). Although tunicamycin was run as a positive control, tunicamycin was not included in the statistical analysis. The expression levels of the individual proteins or genes used to calculate the NF κ B/I κ B and XBP1/XBP1s ratios can be found in the Supporting data (Figure S3). A time course analysis (2, 4, 6, and 24-h) confirmed that melatonin did not

induce changes in TRAF2, I κ B/NF κ B, or XBP1/XBP1s expression at any of these time point (Figure S4). These results indicate that melatonin does not activate the downstream pathways of IRE1 α in BeWo cells. To evaluate ATF6 arm, we assessed ATF6 proteolysis, qualitatively, by Western blot [56, 57] (Figure 3A). Tunicamycin, which induces ER stress by

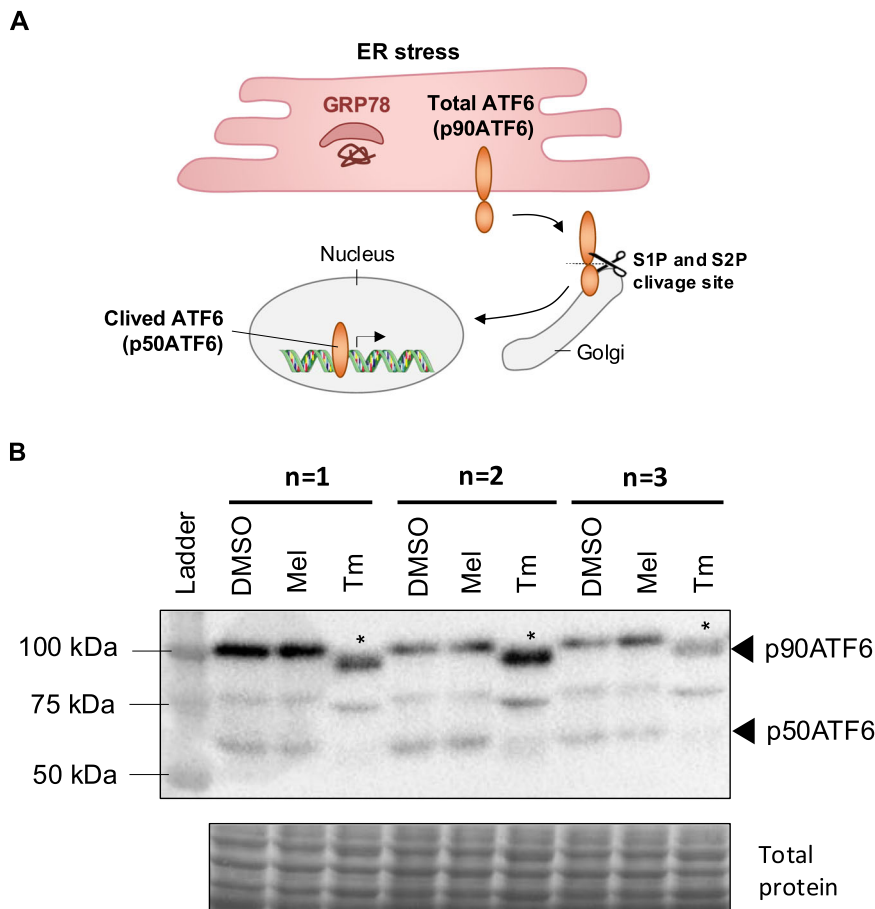


FIGURE 3 | Melatonin has no effect on ATF6 protein cleavage. BeWo cells were treated with the vehicle (DMSO 0.1%) or melatonin (1 mM) or Tm (3.5 $\mu\text{g}/\text{mL}$) under normoxia (8% O_2) conditions during 24-h. (A) Following GRP78 dissociation, ATF6 (p90ATF6, total ATF6) translocates to the Golgi apparatus, where it undergoes proteolytic cleavage by site-1 protease (S1P or MBTPS1) and site-2 protease (S2P or MBTPS2). The resulting activated form, p50ATF6, then enters the nucleus to promote the transcription of genes containing ER stress-response elements (ERSE-1). (B) ATF6 protein cleavage level was determined by Western blot. Equal protein amounts of cell lysates were subjected to Western blot assay using anti-ATF6 antibody. Total protein was used for normalization. ATF6, activating transcription factor 6; DMSO, dimethylsulfoxide; ER, endoplasmic reticulum; GRP78, 78-kDa glucose-regulated protein; Mel, melatonin; S1P, site-1 protease; S2P, site-1 protease; Tm, tunicamycin.

inhibiting protein glycosylation, produced a faster-migrating band of total ATF6 (p90ATF6) compared to the vehicle control and melatonin. This suggests the formation of an unglycosylated ATF6 precursor (p90ATF6*) as reported by other studies [7, 58] (Figure 3B). Tunicamycin treatment led to a reduction in the cleaved form of ATF6 (p50ATF6), suggesting its nuclear translocation and activation—a process not observed with melatonin nor the vehicle control. This supports that tunicamycin activates ATF6, likely via proteolytic cleavage.

3.3 | Melatonin Activates the Pathway of the ER Membrane Transducer Perk

To determine whether melatonin activates the PERK arm of the UPR in BeWo cells, we assessed the protein levels of key downstream factors by Western blot, including Eukaryotic initiation factor 2 subunit alpha (eIF2 α), phosphorylated p-eIF2 α (p-eIF2 α), Activating Transcription Factor 4 (ATF4), C/EBP homologous protein (CHOP), B-cell lymphoma 2 (Bcl-2), B-cell lymphoma 2 associated X (Bax) and Cleaved poly (ADP-ribose) polymerase (cl-Parp) (Figure 4). As with the positive control

tunicamycin, melatonin significantly increased (2.3-fold) the protein level of p-eIF2 α compared to the vehicle control (Figure 4A), without affecting eIF2 α levels (Figure 4B; see p-eIF2 α /eIF2 α ratio in Figure S9). Melatonin significantly up-regulated ATF4 (Figures 4C, 3.3-fold), CHOP (Figures 4D, 2.1-fold) and the proapoptotic protein Bax (Figures 4E, 1.4-fold), while having no effect on the antiapoptotic protein Bcl-2 (Figure 4F). Additionally, melatonin increased cl-Parp protein levels (2.8-fold) compared to vehicle control (Figure 4G). Tunicamycin, included as a positive control, was excluded from the statistical analysis. No effects of melatonin were observed PERK protein levels (Figure S10) or on ATF4 and CHOP gene expression (Figure S11). In summary, melatonin activates the PERK related UPR pathway by increasing p-eIF2 α , ATF4, cl-Parp and Bax proteins levels in BeWo cells.

3.4 | Melatonin Induces Early Apoptosis in BeWo Cells

Instead of restoring homeostasis, UPR pathways trigger apoptosis under persistent ER stress conditions [59]. Melatonin has

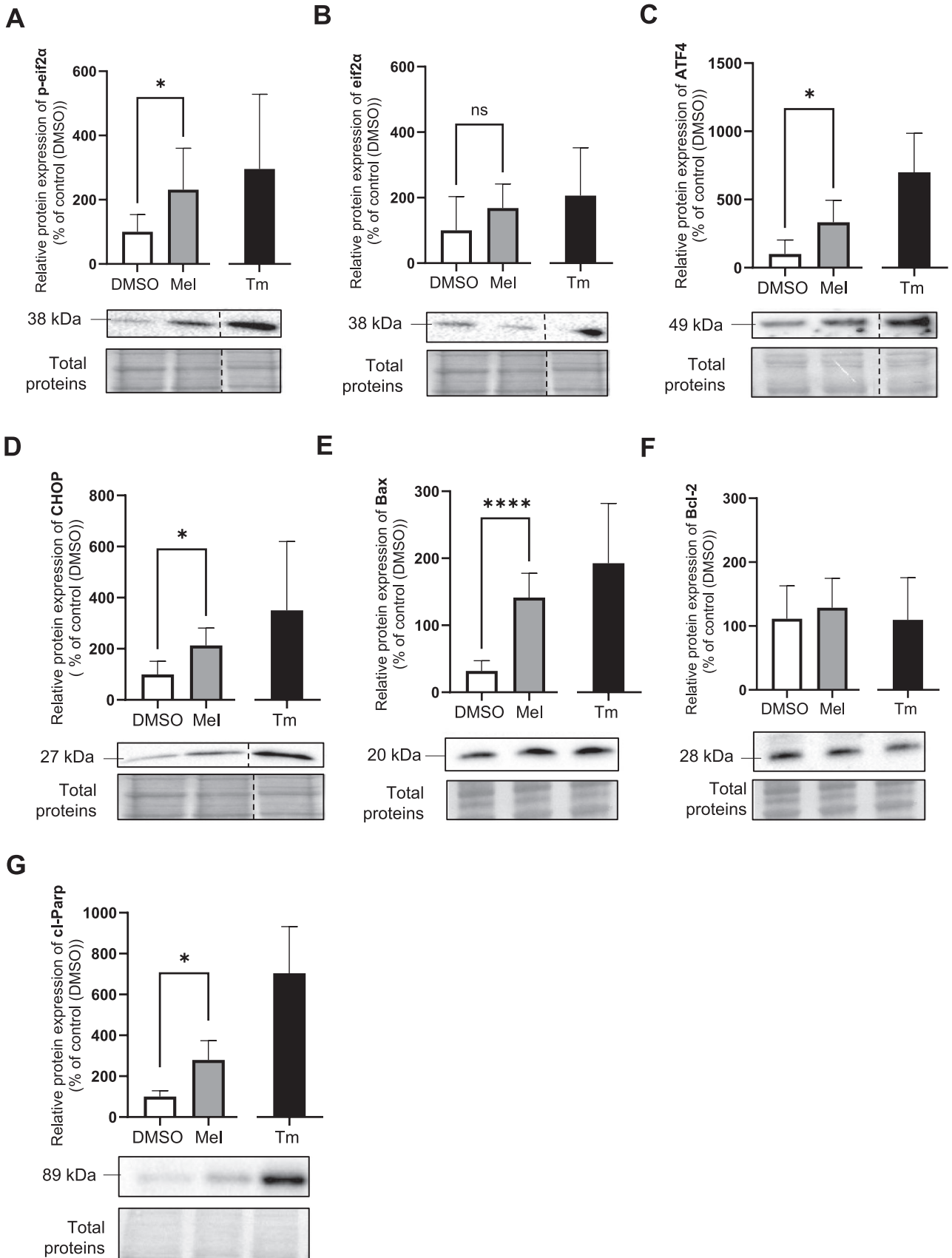


FIGURE 4 | Legend on next page.

been shown to activate UPR pathways, leading to apoptosis in different tumor cells [12, 18, 60]. To investigate its proapoptotic effect in BeWo cells, we performed flow cytometry using Annexin V and PI staining (Figure 5A,B). Compared to the vehicle control, melatonin significantly increased the relative number of Annexin V-positive cells (1.4-fold), indicating activation of early apoptosis. However, melatonin did not affect the number of cells that were positive for both Annexin V and PI, suggesting no effect on late apoptosis. Staurosporine (St) was used as a positive control for apoptosis and confirmed the validity of our approach (Figure S19). These results indicate that melatonin specifically induces early apoptosis in BeWo cells.

3.5 | PERK Knockdown by siRNA Inhibits the Effects of Melatonin on the UPR Signaling Pathways and Early Apoptosis

To determine whether melatonin's effects on the UPR are dependent on PERK, we performed PERK knockdown using siRNA. This approach was more efficient than the chemical inhibitor GSK2656157 (100 nM; 24-h pretreatment), which failed to inhibit p-eIF2 α activation by tunicamycin (Figure S20). PERK knockdown was most effective 3 days posttransfection (Figure S21), leading to a 5.0-fold decrease in PERK protein levels (Figure 6A). While melatonin increased GRP78 (Figure 6B), p-eIF2 α /eIF2 α (Figure 6C,D,E), and ATF4 (Figure 6F) protein levels in scramble siRNA-transfected cells, these effects were abolished in PERK-knockdown cells. These findings suggest that PERK is essential for melatonin to exert its effects on the UPR pathway. To evaluate whether melatonin-induced early apoptosis depends on PERK, we performed flow cytometry using Annexin V and PI staining on BeWo cells transfected with either scrambled siRNA or PERK siRNA (Figure 7A). Melatonin did not affect apoptosis in either condition (Figure 7B–D). Interestingly, PERK knockdown increased early (Figure 7B, 1.7-fold) and total apoptosis (Figure 7D, 1.3-fold) in tunicamycin-treated cells compared to scramble siRNA, suggesting a role for PERK in BeWo cell survival.

4 | Discussion

We have previously demonstrated that melatonin induces cell death in human BeWo choriocarcinoma cells line by inhibiting autophagy, disrupting mitochondrial membrane potential, and decreasing cell viability [35, 38, 40]. Additionally, studies have highlighted the interplay between cell death, the UPR pathway and melatonin in cancer [48, 61]. In this study, we explored melatonin's effect on the UPR in BeWo cells and found that it specifically activates PERK pathway, with no observable effect

on the IRE1 α or ATF6 arms. Consistent with this finding, melatonin treatment also increased GRP78 protein levels, suggesting the initiation of ER stress. Similar findings have been reported in gastric, colorectal, tongue, neck, and head cancers, where melatonin induces the PERK-p-eIF2 α -ATF4 axis [17, 18, 41, 62, 63].

Melatonin treatment increased IRE1 α protein expression in BeWo cells. However, it did not affect *XBP1s/XBP1* gene expression or the protein levels of TRAF2 and NF κ B/I κ B, suggesting that melatonin does not activate this arm of the UPR. Once activated, IRE1 α promotes the splicing of XBP1 mRNA, which supports the ER-associated degradation (ERAD) pathway. In addition, IRE1 α can trigger inflammatory signaling by recruiting TRAF2, which activates the I κ B/NF- κ B pathway [54, 55]. In other cancers where melatonin activates the PERK axis, increased expression of IRE1 α or p-IRE1 α expression has also been observed; however, TRAF2 or XBP1s expression were not assessed [17, 18, 41, 62].

We found that melatonin had no effect on ATF6 in BeWo cells, while other studies reported conflicting results. Some studies suggest inhibitory effects of melatonin on ATF6 cleavage while others indicate elevated ATF6 cleavage in different cancer cells [13, 17, 64]. Our data suggest that PERK is the predominant pathway activated by melatonin in BeWo cells, leading to persistent ER stress and the final stage of the UPR. Under severe or prolonged ER stress, the activity of IRE1 α and ATF6 are attenuated, while the protective activity of PERK shift to a proapoptotic role via CHOP, ultimately leading to cell death [65, 66]. ATF6 and PERK are both activated upon GRP78 dissociation. ATF6 is cleaved in the Golgi to drive transcription of ER stress genes. PERK activation can lead to either phosphorylation of eIF2 α , triggering ATF4-dependent proapoptotic signaling, or activation of the Nuclear factor erythroid 2 related factor 2 (NRF2), which promotes cell survival—depending on the severity and duration of ER stress [54, 67]. Melatonin activation of the p-eIF2 α -ATF4-CHOP proapoptotic pathway suggests a shift away from NRF2 and autophagy-mediated pro-survival response. This aligns with our previous findings, which demonstrated that melatonin inhibits NRF2 and autophagy while increasing proapoptotic factors (Bax, cl-PARP) and reducing antiapoptotic factors (Bcl-2) in BeWo cells [38]. Moreover, PERK knockdown prevented melatonin from activating GRP78, p-eIF2 α , and ATF4, demonstrating that PERK is essential for melatonin's effect on the UPR response. How melatonin interacts with PERK remains unclear. A study showed that pretreatment with luzindole (a melatonin receptor antagonist targeting MT1 and MT2) did not prevent melatonin from enhancing thapsigargin-induced CHOP expression in

FIGURE 4 | Melatonin increases the protein level of factors implicated in PERK-UPR pathway. BeWo cells were treated with the vehicle (DMSO 0.1%) or melatonin (1 mM) or Tm (3.5 μ g/mL) under normoxia (8% O₂) conditions during 24-h. (A) p-eif2 α , (B) eif2 α , (C) ATF4, (D) CHOP, (E) Bax, (F) Bcl-2, and (G) cl-Parp protein level was determined by Western blot. Equal protein amounts of cell lysates (35 μ g) were subjected to Western blot assay using anti-p-eif2 α , anti-eif2 α , anti-ATF4, anti-CHOP, anti-Bax, anti-Bcl-2 and anti-cl-Parp. Total protein was used for normalization. ATF4, activating transcription factor 4; Bax, Bcl-2-associated X; Bcl-2, B-cell lymphoma 2; CHOP, DNA damage-inducible transcript 3, also known as C/EBP homologous protein; cl-Parp, cleaved poly (ADP-ribose) polymerase; DMSO, dimethylsulfoxide; eif2 α , eukaryotic initiation factor 2 subunit alpha; Mel, melatonin; p-eif2 α , phosphorylation of the eukaryotic initiation factor 2 subunit alpha; Tm, tunicamycin. Data are shown as mean \pm SD and were analyzed using an unpaired *t*-test (DMSO vs. Mel, **p* < 0.05), *n* = 4–6. To facilitate readers' experiences, the band order of certain blots has been changed. Those modifications have been identified with dotted lines. Original western blots are included under Figures S12–S18.

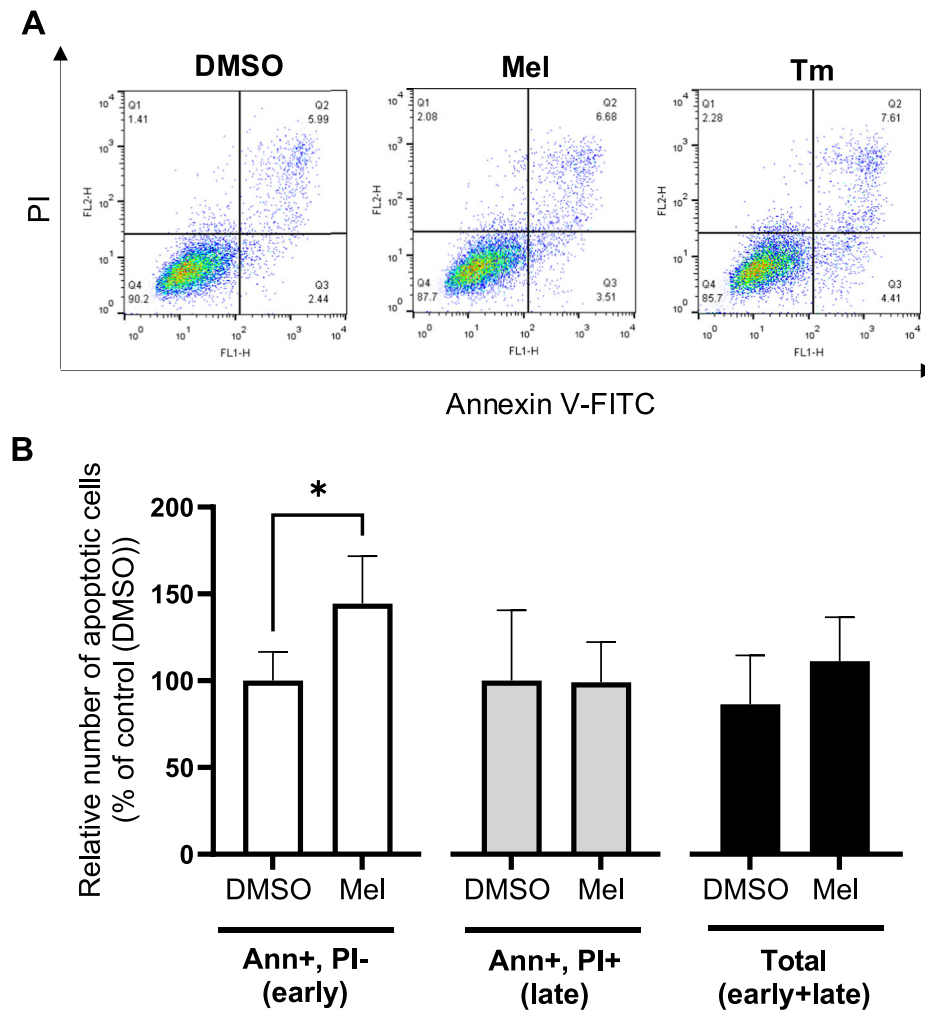


FIGURE 5 | Melatonin increases early apoptosis in BeWo cells. BeWo cells were treated with the vehicle (DMSO) or melatonin (1 mM) or Tm (3.5 $\mu\text{g}/\text{mL}$) under normoxia (8% O_2) conditions during 24-h. (A) Number of apoptotic cells was determined by FACS using Annexin V and PI staining. (B) Number of early or late apoptotic cells are expressed as a percentage of the control DMSO. Ann, annexin V; DMSO, dimethylsulfoxide; Early, early apoptosis; Late, late apoptosis; Mel, melatonin; PI, propidium iodide; Tm, tunicamycin; Total, total apoptosis. Data are shown as mean \pm SD and were analyzed using an unpaired *t*-test (DMSO vs. Mel, * $p < 0.05$), $n = 7$.

renal cancer cells, suggesting a potential receptor-independent mechanism [68]. Similarly, Lang et al. (2021) proposed that melatonin activates the PERK-ATF4 pathway independently of its receptors, as MT1 was absent in their head and neck cancer models [62]. However, other studies suggest that melatonin may act through receptor-dependent mechanisms [62, 69, 70]. These findings highlight a complex interplay between melatonin and the PERK pathway, warranting further investigation.

We observed that melatonin induces early apoptosis in BeWo cells but has no effect on late apoptosis. The proapoptotic effects of melatonin have been evaluated in various cancer cell lines using flow cytometry with Annexin V-PI staining [13, 18, 63, 71–83]. While some studies reported an increase in total apoptosis, they did not distinguish between early and late apoptotic stages [13, 18, 63, 71–75]. Other studies that have examined both early and late apoptosis report inconsistent findings potentially due to difference in cell types, melatonin concentration and exposure duration [76–83]. In fact, in neuroblastoma cells, melatonin promotes early apoptosis and induces differentiation, reducing proliferation and the initial stages of

carcinogenesis [84]. Annexin V binds to externalized phosphatidylserine, an early marker of apoptosis and a process essential for cell differentiation [85]. Previous work has shown that melatonin regulates Human chorionic gonadotropin beta chain (hCG- β) secretion in BeWo cells, a key factor in trophoblast differentiation [20, 86, 87]. The initiation of early apoptosis and hCG- β regulation by melatonin may suggest a role in modulating BeWo cell differentiation and affecting tumor progression. More research needs to be done on the subject.

Melatonin has been shown to trigger different apoptotic signaling pathways depending on the cancer model [47]. For example, Li et al. (2022) reported that melatonin's proapoptotic actions occur through NF- κB and I κB modulation in gastric cancer cells, alongside involvement of the PERK pathway [63]. In contrast, our data suggest that in BeWo trophoblastic cells, melatonin's effects are more specifically associated with the PERK pathway, rather than with NF- κB or I κB . This contrast underscores the need to better understand the mechanisms behind melatonin's proapoptotic effects, especially in specific cancer models pathway. Our previous work has shown that

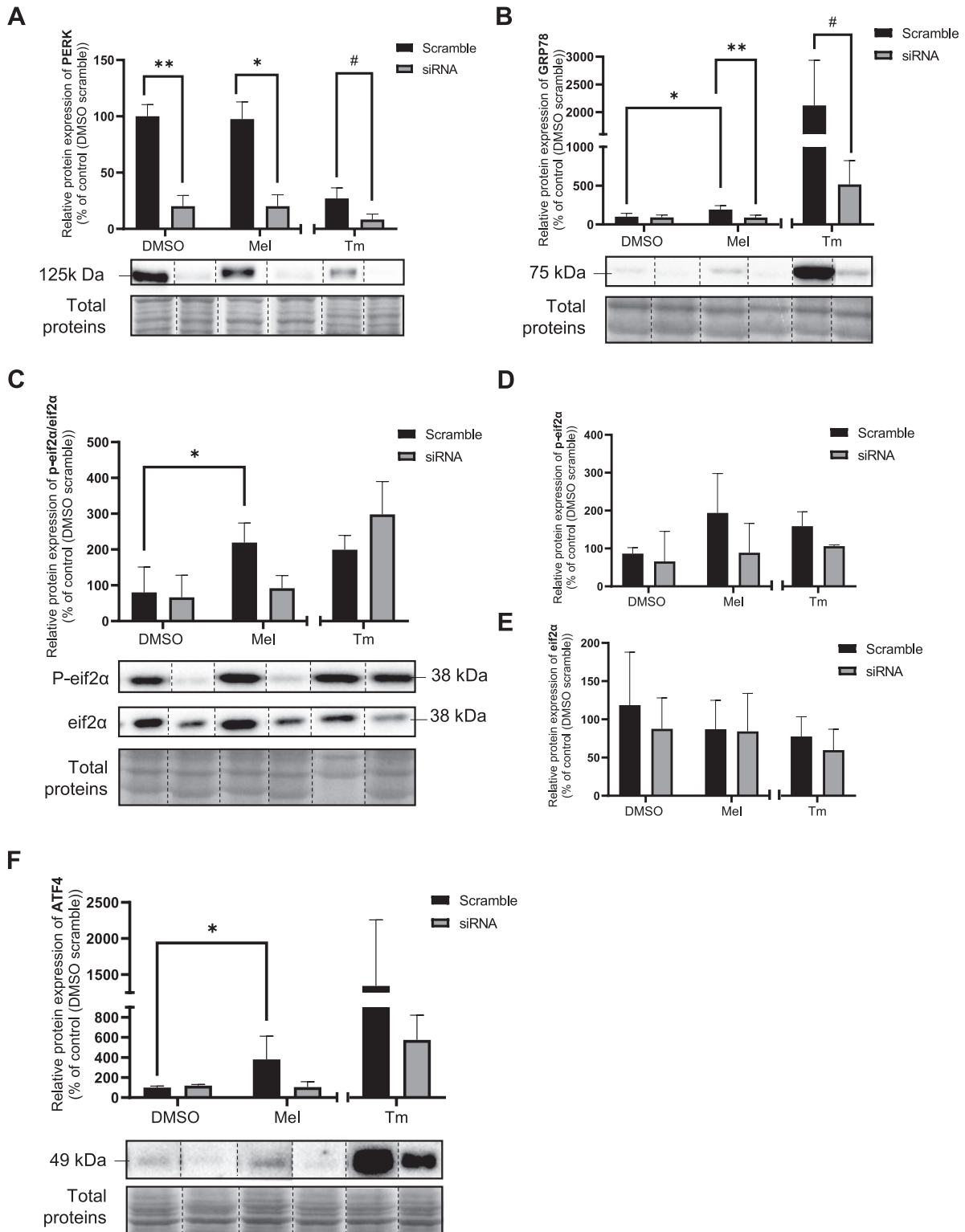


FIGURE 6 | Knockdown of PERK inhibits the ability of melatonin to induce PERK-UPR pathway. BeWo cells subjected to scramble (SC) or siRNA against PERK for 72-h were treated with the vehicle (DMSO 0.1%) or melatonin (1 mM) or TM (3.5 μ g/mL) under normoxia (8% O₂) conditions during 24-h. (A) PERK, (B) GRP78, (C) p-eif2 α /eif2 α ratio (D) p-eif2 α , (E) eif2 α , and (F) ATF4 protein level was determined by Western blot. Equal protein amounts of cell lysates (35 μ g) were subjected to Western blot assay using anti-PERK, anti-GRP78, anti-p-eif2 α , anti-eif2 α ratio and anti-ATF4. Total protein was used for normalization. ATF4, activating transcription factor 4; DMSO, dimethylsulfoxide; eif2 α , eukaryotic initiation factor 2 subunit alpha; GRP78, 78-kDa glucose-regulated protein; Mel, melatonin; p-eif2 α , phosphorylation of the eukaryotic initiation factor 2 subunit alpha; PERK, protein kinase R (PKR)-like; Tm, tunicamycin. Data are shown as mean \pm SD and were analyzed using ANOVA followed by Tukey post hoc test (* p < 0.05), or an unpaired t -test (SC Tm vs. si Tm, # p < 0.05, ## p < 0.01), n = 5. To facilitate readers' experiences, the band order has been changed. Those modifications have been identified with dotted lines. Original western blots are included under Figures S22–S26.

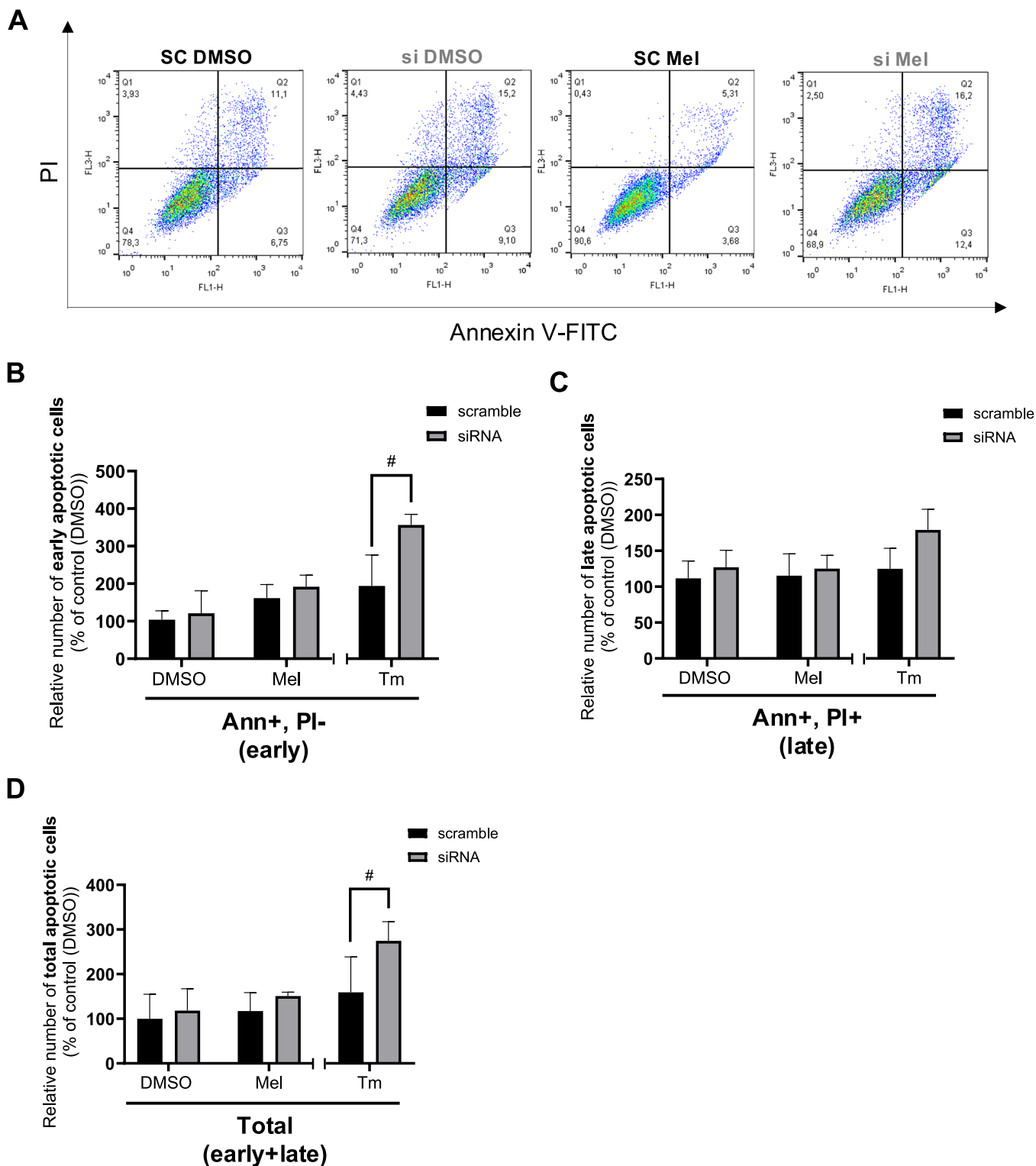


FIGURE 7 | Melatonin and knockdown of PERK have no effect on early and late apoptosis. BeWo cells subjected to scramble (SC) or siRNA against PERK (si) for 72-h were treated with the vehicle (DMSO 0.1%) or melatonin (1 mM) or Tm (3.5 μ g/mL) under 8% of oxygen during 24-h. (A) Apoptotic cells were determined by FACS using Annexin V and PI. Number of (B) early, (C) late or (D) total apoptotic cells are expressed as a percentage of control DMSO. Ann, annexin V; DMSO, dimethylsulfoxide; Early, early apoptosis; Late, late apoptosis; Mel, melatonin; PERK, (PKR)-like protein kinase R; PI, propidium iodide; Tm, tunicamycin; Total, total (early + late) apoptosis. Data are shown as mean \pm SD and were analyzed using ANOVA followed by Tukey post hoc test ($*p < 0.05$), or an unpaired *t*-test (SC Tm vs si Tm, [#] $p < 0.05$, ^{##} $p < 0.01$). $n = 5$.

melatonin contributes to BeWo cell death by inducing intrinsic apoptosis and downregulating autophagy [35, 38]. Consistent with these findings, the present study shows that melatonin increases the expression of Bax, a key protein in intrinsic

apoptosis, as well as cl-PARP. The apoptotic effect of melatonin is mediated through the PERK-CHOP pathway in various carcinoma cell types [33, 43]. CHOP induces apoptosis by modulating Bcl-2 and Bax levels [88, 89]. In our study, the induction

of early apoptosis by melatonin was lost when using PERK-specific siRNA or scramble siRNA, suggesting that transfection may have induced a protective pathway in BeWo cells. For example, siRNA transfection can activate autophagy that may help sustain cell viability [90].

Both the presence of melatonin and the absence of PERK appear detrimental to BeWo choriocarcinoma cells. Knockdown of PERK increased early and total apoptosis in BeWo cells treated with tunicamycin compared to scramble siRNA-transfected cells. This could be due to PERK deficiency leading to increased ROS accumulation and reduced NRF2 activation [91–93]. As tunicamycin induces oxidative stress, the absence of PERK may lead to reduced NRF2 activation, impairing the ability of BeWo cells to cope with the oxidative challenge. This could explain why PERK appears to contribute to BeWo cell survival, as observed in other cancer types [91–93]. PERK has been associated with poor prognosis in breast and thyroid cancers, potentially influencing tumor microenvironment and immune cell infiltration [94]. Suppression of PERK signaling has been shown to impede tumor growth and increase tumor sensibility to chemotherapy in colon cancer [95]. In colon and lung cancer, activation of p-eIF2 α and ATF4 signaling increases resistance to chemotherapy [96, 97]. More specifically, ATF4 overexpression leads to multidrug resistance, while ATF4 knockout reduces glutathione synthesis and increases chemotherapy sensitivity in lung cancer [97]. ER stress appears to be a pathway that enables cancer cells to resist apoptosis induced by chemotherapeutic agents. In support of this, PERK inhibitors have been shown to suppress tumor development [61]. Melatonin may also help attenuate this resistance by inhibiting PERK pathway [98]. Fan et al. demonstrated that pretreatment with tunicamycin and melatonin increased doxorubicin-induced apoptosis in hepatic carcinoma cells, whereas tunicamycin alone reduces doxorubicin-induced apoptosis in liver cancer cells [98]. Other studies have shown that melatonin enhances cytotoxic effects of chemotherapeutic agents in cancer cells, increasing hepatocellular carcinoma sensitivity to sorafenib via the PERK-ATF4-Beclin1 pathway [33, 99].

5 | Conclusion

This study demonstrates that melatonin activates the PERK arm of the UPR pathway and induces early apoptosis in BeWo cells. Moreover, PERK deficiency increases the susceptibility of these cells to apoptosis. Our findings highlight the role of melatonin and the PERK-UPR pathway in promoting cancer cell apoptosis. Targeting UPR signaling to disrupt the balance between adaptive ER stress and ER stress-induced apoptosis through melatonin could offer a novel therapeutic approach for treating cancers, including choriocarcinoma. Future studies should explore the potential of melatonin as an adjuvant therapy, potentially amplifying ER stress and apoptosis to enhance therapeutic efficacy in cancer treatment while protecting healthy cells.

Author Contributions

All co-authors contributed to the content and discussion of each section of the manuscript. Josianne Bienvenue-Pariseault and Cathy Vaillancourt contributed to the design of the study protocol. Josianne

Bienvenue-Pariseault, Philippe Wong-Yen, and Darius Stakamatos were involved in data acquisition. Lucas Sagrillo-Fagundes, Marie Cohen and Cathy Vaillancourt were actively involved in the revising of the manuscript. Josianne Bienvenue-Pariseault wrote the original version and created the figures of the manuscript. All co-authors provided critical revisions and their final approval for the manuscript's publication and all authors agree with the contents of this manuscript.

Acknowledgments

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) (06778-2019) to C.V. NSERC Graduate Scholarship Doctoral Program and Fonds de recherche du Québec Nature et Technologie (FRQNT) doctoral training scholarships studentships to JBP and LSG and Undergraduate Student Research Awards for DM received a studentship from Foundation Armand Frappier and from the NSERC Undergraduate Student Research Award studentship.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data available on request from the authors.

References

1. D. S. Schwarz and M. D. Blower, "The Endoplasmic Reticulum: Structure, Function and Response to Cellular Signaling," *Cellular and Molecular Life Sciences* 73, no. 1 (2016): 79–94, <https://doi.org/10.1007/s00018-015-2052-6>.
2. P. Jiang, M. Gan, W.-L. Lin, and S. H. Yen, "Nutrient Deprivation Induces α -Synuclein Aggregation Through Endoplasmic Reticulum Stress Response and SREBP2 Pathway," *Frontiers in Aging Neuroscience* 6 (2014): 268, <https://doi.org/10.3389/fnagi.2014.00268>.
3. V. Sharma, R. Kaur, A. Bhatnagar, and J. Kaur, "Low-pH-Induced Apoptosis: Role of Endoplasmic Reticulum Stress-Induced Calcium Permeability and Mitochondria-Dependent Signaling," *Cell Stress and Chaperones* 20, no. 3 (2015): 431–440, <https://doi.org/10.1007/s12192-014-0568-6>.
4. L. Guan, R. Ge, and S. Ma, "Newsights of Endoplasmic Reticulum in Hypoxia," *Biomedicine & Pharmacotherapy = Biomedicine & pharmacotherapie* 175 (2024): 116812, <https://doi.org/10.1016/j.biopha.2024.116812>.
5. W. Tirasophon, A. A. Welihinda, and R. J. Kaufman, "A Stress Response Pathway From the Endoplasmic Reticulum to the Nucleus Requires a Novel Bifunctional Protein Kinase/Endoribonuclease (Ire1p) in Mammalian Cells," *Genes & Development* 12, no. 12 (1998): 1812–1824.
6. H. P. Harding, Y. Zhang, and D. Ron, "Protein Translation and Folding Are Coupled by an Endoplasmic-Reticulum-Resident Kinase," *Nature* 397, no. 6716 (1999): 271–274, <https://doi.org/10.1038/16729>.
7. K. Haze, H. Yoshida, H. Yanagi, T. Yura, and K. Mori, "Mammalian Transcription Factor ATF6 Is Synthesized as a Transmembrane Protein and Activated by Proteolysis in Response to Endoplasmic Reticulum Stress," *Molecular Biology of the Cell* 10, no. 11 (1999): 3787–3799.
8. C. Xu, "Endoplasmic Reticulum Stress," *Journal of Clinical Investigation* 115, no. 10 (2005): 2656–2664, <https://doi.org/10.1172/jci26373>.
9. C. M. Osowski and F. Urano, "The Binary Switch Between Life and Death of Endoplasmic Reticulum-Stressed β Cells," *Current Opinion in Endocrinology, Diabetes & Obesity* 17, no. 2 (2010): 107–112, <https://doi.org/10.1097/MED.0b013e3283372843>.
10. A. Papaioannou and E. Chevet, "Driving Cancer Tumorigenesis and Metastasis Through UPR Signaling." in *Coordinating Organismal*

- Physiology Through the Unfolded Protein Response*, eds. R. L. Wiseman and C. M. Haynes. Springer International Publishing, 2018), 159–192.
11. J.-Y. Tang, F. Ou-Yang, M.-F. Hou, et al., “Oxidative Stress-Modulating Drugs Have Preferential Anticancer Effects - Involving the Regulation of Apoptosis, DNA Damage, Endoplasmic Reticulum Stress, Autophagy, Metabolism, and Migration,” *Seminars in Cancer Biology* 58 (2019): 109–117, <https://doi.org/10.1016/j.semcancer.2018.08.010>.
 12. Y. Zheng, J. Tu, X. Wang, et al., “The Therapeutic Effect of Melatonin on GC by Inducing Cell Apoptosis and Autophagy Induced by Endoplasmic Reticulum Stress,” *OncoTargets and Therapy* 12 (2019): 10187–10198, <https://doi.org/10.2147/ott.S226140>.
 13. S. Liu, B. Liang, H. Jia, Y. Jiao, Z. Pang, and Y. Huang, “Evaluation of Cell Death Pathways Initiated by Antitumor Drugs Melatonin and Valproic Acid in Bladder Cancer Cells,” *FEBS Open Bio* 7, no. 6 (2017): 798–810, <https://doi.org/10.1002/2211-5463.12223>.
 14. L. Zha, L. Fan, G. Sun, et al., “Melatonin Sensitizes Human Hepatoma Cells to Endoplasmic Reticulum Stress-Induced Apoptosis,” *Journal of Pineal Research* 52, no. 3 (2012): 322–331, <https://doi.org/10.1111/j.1600-079X.2011.00946.x>.
 15. B. Zhou, Q. Lu, J. Liu, et al., “Melatonin Increases the Sensitivity of Hepatocellular Carcinoma to Sorafenib Through the PERK-ATF4-Beclin1 Pathway,” *International Journal of Biological Sciences* 15, no. 9 (2019): 1905–1920, <https://doi.org/10.7150/ijbs.32550>.
 16. H. S. Kim, T.-J. Kim, and Y.-M. Yoo, “Melatonin Combined With Endoplasmic Reticulum Stress Induces Cell Death via the PI3K/Akt/mTOR Pathway in B16F10 Melanoma Cells,” *PLoS One* 9, no. 3 (2014): e92627, <https://doi.org/10.1371/journal.pone.0092627>.
 17. H. Liu, Y. Zheng, S. Kan, et al., “Melatonin Inhibits Tongue Squamous Cell Carcinoma: Interplay of ER Stress-Induced Apoptosis and Autophagy With Cell Migration,” *Heliyon* 10, no. 8 (2024): e29291, <https://doi.org/10.1016/j.heliyon.2024.e29291>.
 18. C. W. Yun, S. Kim, J. H. Lee, and S. H. Lee, “Melatonin Promotes Apoptosis of Colorectal Cancer Cells via Superoxide-Mediated ER Stress by Inhibiting Cellular Prion Protein Expression,” *Anticancer Research* 38, no. 7 (2018): 3951–3960, <https://doi.org/10.21873/anticancer.12681>.
 19. N. Zisapel, “New Perspectives on the Role of Melatonin in Human Sleep, Circadian Rhythms and Their Regulation,” *British Journal of Pharmacology* 175, no. 16 (2018): 3190–3199, <https://doi.org/10.1111/bph.14116>.
 20. D. Lanoix, H. Beghdadi, J. Lafond, and C. Vaillancourt, “Human Placental Trophoblasts Synthesize Melatonin and Express Its Receptors,” *Journal of Pineal Research* 45, no. 1 (2008): 50–60, <https://doi.org/10.1111/j.1600-079X.2008.00555.x>.
 21. D. Acuña-Castroviejo, G. Escames, C. Venegas, et al., “Extrapineal Melatonin: Sources, Regulation, and Potential Functions,” *Cellular and Molecular Life Sciences: CMLS* 71, no. 16 (2014): 2997–3025, <https://doi.org/10.1007/s00018-014-1579-2>.
 22. S. M. Nabavi, S. F. Nabavi, A. Sureda, et al., “Anti-Inflammatory Effects of Melatonin: A Mechanistic Review,” supplement, *Critical Reviews in Food Science and Nutrition* 59, no. sup 1 (2019): S4–S16, <https://doi.org/10.1080/10408398.2018.1487927>.
 23. A. Galano, D. X. Tan, and R. J. Reiter, “Melatonin as a Natural Ally Against Oxidative Stress: A Physicochemical Examination,” *Journal of Pineal Research* 51, no. 1 (2011): 1–16, <https://doi.org/10.1111/j.1600-079X.2011.00916.x>.
 24. M. Rafiyan, R. J. Reiter, S. M. Rasooli Manesh, et al., “Programmed Cell Death and Melatonin: A Comprehensive Review,” *Functional & Integrative Genomics*. 24, no. 5 (2024): 169, <https://doi.org/10.1007/s10142-024-01454-4>.
 25. R. J. Reiter, S. A. Rosales-Corral, D. X. Tan, et al., “Melatonin, a Full Service Anti-Cancer Agent: Inhibition of Initiation, Progression and Metastasis,” *International Journal of Molecular Sciences* 18, no. 4 (2017): 843, <https://doi.org/10.3390/ijms18040843>.
 26. V. Pourbarkhordar, S. Rahmani, A. Roohbakhsh, A. W. Hayes, and G. Karimi, “Melatonin Effect on Breast and Ovarian Cancers by Targeting the PI3K/Akt/mTOR Pathway,” *IUBMB Life* 76, no. 12 (2024): 1035–1049, <https://doi.org/10.1002/iub.2900>.
 27. C. Martínez-Campa, V. Álvarez-García, C. Alonso-González, A. González, and S. Cos, “Melatonin and Its Role in the Epithelial-to-Mesenchymal Transition (EMT) in Cancer,” *Cancers* 16, no. 5 (2024): 956.
 28. N. H. Goradel, M. H. Asghari, M. Moloudizargari, B. Negahdari, H. Haghi-Aminjan, and M. Abdollahi, “Melatonin as An Angiogenesis Inhibitor to Combat Cancer: Mechanistic Evidence,” *Toxicology and Applied Pharmacology* 335 (2017): 56–63, <https://doi.org/10.1016/j.taap.2017.09.022>.
 29. M. Samec, A. Liskova, L. Koklesova, et al., “Metabolic Anti-Cancer Effects of Melatonin: Clinically Relevant Prospects,” *Cancers* 13, no. 12 (2021): 3018.
 30. K. Mortezaee, M. Najafi, B. Farhood, et al., “Modulation of Apoptosis by Melatonin for Improving Cancer Treatment Efficiency: An Updated Review,” *Life Sciences* 228 (2019): 228–241, <https://doi.org/10.1016/j.lfs.2019.05.009>.
 31. R. Hardeland, “Melatonin and Inflammation—Story of a Double-Edged Blade,” *Journal of Pineal Research* 65, no. 4 (2018): e12525, <https://doi.org/10.1111/jpi.12525>.
 32. N. Das, S. Mukherjee, A. Das, P. Gupta, A. Bandyopadhyay, and S. Chattopadhyay, “Intra-Tumor ROS Amplification by Melatonin Interferes in the Apoptosis-Autophagy-Inflammation-EMT Collusion in the Breast Tumor Microenvironment,” *Heliyon* 10, no. 1 (2024): e23870, <https://doi.org/10.1016/j.heliyon.2023.e23870>.
 33. S. Mehrzadi, M. H. Pourhanifeh, A. Mirzaei, F. Moradian, and A. Hosseinzadeh, “An Updated Review of Mechanistic Potentials of Melatonin Against Cancer: Pivotal Roles in Angiogenesis, Apoptosis, Autophagy, Endoplasmic Reticulum Stress and Oxidative Stress,” *Cancer Cell International* 21, no. 1 (2021): 188, <https://doi.org/10.1186/s12935-021-01892-1>.
 34. S. Malhotra, G. Sawhney, and P. Pandhi, “The Therapeutic Potential of Melatonin: A Review of the Science,” *MedGenMed: Medscape General Medicine*. 6, no. 2 (2004): 46.
 35. D. Lanoix, A.-A. Lacasse, R. J. Reiter, and C. Vaillancourt, “Melatonin: The Smart Killer,” *Molecular and Cellular Endocrinology* 348, no. 1 (2012): 1–11, <https://doi.org/10.1016/j.mce.2011.08.025>.
 36. S. Rohilla, M. Singh, S. Priya, et al., “Exploring the Mechanical Perspective of a New Anti-Tumor Agent: Melatonin,” *Journal of Environmental Pathology, Toxicology and Oncology* 42, no. 1 (2023): 1–16, <https://doi.org/10.1615/JEnvironPatholToxicolOncol.2022042088>.
 37. X. Liu, Z. Chen, C. C. Chua, et al., “Melatonin as an Effective Protector Against Doxorubicin-Induced Cardiotoxicity,” *American Journal of Physiology-Heart and Circulatory Physiology* 283, no. 1 (2002): H254–H263, <https://doi.org/10.1152/ajpheart.01023.2001>.
 38. L. Sagrillo-Fagundes, J. Bienvenue-Pariseault, and C. Vaillancourt, “Melatonin: The Smart Molecule That Differentially Modulates Autophagy in Tumor and Normal Placental Cells,” *PLoS One* 14, no. 1 (2019): e0202458, <https://doi.org/10.1371/journal.pone.0202458>.
 39. D. Lanoix, A. A. Lacasse, R. J. Reiter, and C. Vaillancourt, “Melatonin: The Watchdog of Villous Trophoblast Homeostasis Against Hypoxia/Reoxygenation-Induced Oxidative Stress and Apoptosis,” *Molecular and Cellular Endocrinology* 381, no. 1–2 (2013): 35–45, <https://doi.org/10.1016/j.mce.2013.07.010>.
 40. L. Sagrillo-Fagundes, E. M. Assunção Salustiano, R. Ruano, R. P. Markus, and C. Vaillancourt, “Melatonin Modulates Autophagy and Inflammation Protecting Human Placental Trophoblast From Hypoxia/Reoxygenation,” *Journal of Pineal Research* 65, no. 4 (2018): e12520, <https://doi.org/10.1111/jpi.12520>.

41. K. C. Chok, R. Y. Koh, M. G. Ng, P. Y. Ng, and S. M. Chye, "Melatonin Induces Autophagy via Reactive Oxygen Species-Mediated Endoplasmic Reticulum Stress Pathway in Colorectal Cancer Cells," *Molecules* 26, no. 16 (2021): 5038.
42. M. H. Pourhanifeh, M. Sharifi, R. J. Reiter, A. Davoodabadi, and Z. Asemi, "Melatonin and Non-Small Cell Lung Cancer: New Insights Into Signaling Pathways," *Cancer Cell International* 19 (2019): 131, <https://doi.org/10.1186/s12935-019-0853-7>.
43. A. J. Moreira, R. Ordoñez, C. T. Cerski, et al., "Melatonin Activates Endoplasmic Reticulum Stress and Apoptosis in Rats With Diethylnitrosamine-Induced Hepatocarcinogenesis," *PLoS One* 10, no. 12 (2015): e0144517, <https://doi.org/10.1371/journal.pone.0144517>.
44. R. Shafabakhsh, R. J. Reiter, H. Mirzaei, S. N. Teymoordash, and Z. Asemi, "Melatonin: A New Inhibitor Agent for Cervical Cancer Treatment," *Journal of Cellular Physiology* 234, no. 12 (2019): 21670–21682, <https://doi.org/10.1002/jcp.28865>.
45. T. Chawla, G. Bouchard-Fortier, G. Turashvili, R. Osborne, K. Hack, and P. Glanc, "Gestational Trophoblastic Disease: An Update," *Abdominal Radiology*. 48, no. 5 (2023): 1793–1815, <https://doi.org/10.1007/s00261-023-03820-5>.
46. T. Wang, W. Guo, X. Ren, et al., "Progress of Immunotherapies in Gestational Trophoblastic Neoplasms," *Journal of Cancer Research and Clinical Oncology* 149, no. 16 (2023): 15275–15285, <https://doi.org/10.1007/s00432-023-05010-8>.
47. L. G. de Almeida Chuffa, F. R. F. Seiva, H. S. Silveira, et al., "Melatonin Regulates Endoplasmic Reticulum Stress in Diverse Pathophysiological Contexts: A Comprehensive Mechanistic Review," *Journal of Cellular Physiology* 239, no. 11 (2024): e31383, <https://doi.org/10.1002/jcp.31383>.
48. W. Zhang, Y. Shi, L. Oyang, et al., "Endoplasmic Reticulum Stress—A Key Guardian in Cancer," *Cell Death Discovery* 10, no. 1 (2024): 343, <https://doi.org/10.1038/s41420-024-02110-3>.
49. L. Sagrillo-Fagundes, H. Clabault, L. Laurent, et al., "Human Primary Trophoblast Cell Culture Model to Study the Protective Effects of Melatonin Against Hypoxia/Reoxygenation-Induced Disruption," *Journal of Visualized Experiments*, no. 113 (2016): e54228, <https://doi.org/10.3791/54228>.
50. S. C. Taylor, T. Berkelman, G. Yadav, and M. Hammond, "A Defined Methodology for Reliable Quantification of Western Blot Data," *Molecular Biotechnology*. 55, no. 3 (2013): 217–226, <https://doi.org/10.1007/s12033-013-9672-6>.
51. S. C. Taylor, L. K. Rosselli-Murai, B. Crobeddu, and I. Plante, "A Critical Path to Producing High Quality, Reproducible Data From Quantitative Western Blot Experiments," *Scientific Reports* 12, no. 1 (2022): 17599, <https://doi.org/10.1038/s41598-022-22294-x>.
52. S. Taylor, M. Wakem, G. Dijkman, M. Alsarraj, and M. Nguyen, "A Practical Approach to RT-qPCR—Publishing Data That Conform to the MIQE Guidelines," *Methods* 50, no. 4 (2010): S1–S5, <https://doi.org/10.1016/j.ymeth.2010.01.005>.
53. S. A. Bustin, V. Benes, J. A. Garson, et al., "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments," *Clinical Chemistry* 55, no. 4 (2009): 611–622, <https://doi.org/10.1373/clinchem.2008.112797>.
54. P. Walter and D. Ron, "The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation," *Science* 334, no. 6059 (2011): 1081–1086, <https://doi.org/10.1126/science.1209038>.
55. S. Bashir, M. Banday, O. Qadri, et al., "The Molecular Mechanism and Functional Diversity of UPR Signaling Sensor IRE1," *Life Sciences* 265 (2021): 118740. <https://doi.org/10.1016/j.lfs.2020.118740>.
56. A. H. Lee, N. N. Iwakoshi, and L. H. Glimcher, "XBP-1 Regulates a Subset of Endoplasmic Reticulum Resident Chaperone Genes in the Unfolded Protein Response," *Molecular and Cellular Biology* 23, no. 21 (2003): 7448–7459, <https://doi.org/10.1128/mcb.23.21.7448-7459.2003>.
57. K. Yamamoto, T. Sato, T. Matsui, et al., "Transcriptional Induction of Mammalian ER Quality Control Proteins Is Mediated by Single or Combined Action of ATF6 α and XBP1," *Developmental Cell*. 13, no. 3 (2007): 365–376, <https://doi.org/10.1016/j.devcel.2007.07.018>.
58. M. Li, P. Baumeister, B. Roy, et al., "ATF6 as a Transcription Activator of the Endoplasmic Reticulum Stress Element: Thapsigargin Stress-Induced Changes and Synergistic Interactions With NF-Y and YY1," *Molecular and Cellular Biology* 20, no. 14 (2000): 5096–5106, <https://doi.org/10.1128/mcb.20.14.5096-5106.2000>.
59. E. Szegezdi, S. E. Logue, A. M. Gorman, and A. Samali, "Mediators of Endoplasmic Reticulum Stress-Induced Apoptosis," *EMBO Reports* 7, no. 9 (2006): 880–885, <https://doi.org/10.1038/sj.embor.7400779>.
60. A. Fernández, R. Ordóñez, R. J. Reiter, J. González-Gallego, and J. L. Mauriz, "Melatonin and Endoplasmic Reticulum Stress: Relation to Autophagy and Apoptosis," *Journal of Pineal Research* 59, no. 3 (2015): 292–307, <https://doi.org/10.1111/jpi.12264>.
61. G. Bonsignore, S. Martinotti, and E. Ranzato, "Endoplasmic Reticulum Stress and Cancer: Could Unfolded Protein Response Be a Druggable Target for Cancer Therapy?," *International Journal of Molecular Sciences* 24, no. 2 (2023): 1566, <https://doi.org/10.3390/ijms24021566>.
62. L. Lang, Y. Xiong, N. Prieto-Dominguez, et al., "FGF19/FGFR4 Signaling Axis Confines and Switches the Role of Melatonin in Head and Neck Cancer Metastasis," *Journal of Experimental & Clinical Cancer Research* 40, no. 1 (2021): 93, <https://doi.org/10.1186/s13046-021-01888-9>.
63. W. Li, C. Hu, X. Zhong, J. Wu, and G. Li, "Melatonin Induces AGS Gastric Cancer Cell Apoptosis via Regulating PERK/eIF2 α and HSF1/NF- κ B Signaling Pathway," *Annals of Clinical and Laboratory Science* 52, no. 1 (2022): 40–47.
64. L. J. Bu, H. Q. Yu, L. L. Fan, et al., "Melatonin, a Novel Selective ATF-6 Inhibitor, Induces Human Hepatoma Cell Apoptosis Through COX-2 Downregulation," *World Journal of Gastroenterology* 23, no. 6 (2017): 986–998, <https://doi.org/10.3748/wjg.v23.i6.986>.
65. J. H. Lin, H. Li, D. Yasumura, et al., "IRE1 Signaling Affects Cell Fate During the Unfolded Protein Response," *Science (New York, N.Y.)* 318, no. 5852 (2007): 944–949, <https://doi.org/10.1126/science.1146361>.
66. R. Jäger, M. J. M. Bertrand, A. M. Gorman, P. Vandenabeele, and A. Samali, "The Unfolded Protein Response at the Crossroads of Cellular Life and Death During Endoplasmic Reticulum Stress," *Biology of the Cell* 104, no. 5 (2012): 259–270, <https://doi.org/10.1111/boc.201100055>.
67. M. Márton, A. Kurucz, B. Lizák, É. Margittai, G. Bánhegyi, and O. Kapuy, "A Systems Biological View of Life-and-Death Decision With Respect to Endoplasmic Reticulum Stress-The Role of PERK Pathway," *International Journal of Molecular Sciences* 18, no. 1 (2017): 58, <https://doi.org/10.3390/ijms18010058>.
68. K. Min, H. S. Kim, E. J. Park, and T. K. Kwon, "Melatonin Enhances Thapsigargin-Induced Apoptosis Through Reactive Oxygen Species-Mediated Upregulation of CCAAT-Enhancer-Binding Protein Homologous Protein in Human Renal Cancer Cells," *Journal of Pineal Research*. 53, no. 1 (2012): 99–106, <https://doi.org/10.1111/j.1600-079X.2012.00975.x>.
69. C. Rodríguez-Santana, J. Florido, L. Martínez-Ruiz, A. López-Rodríguez, D. Acuña-Castroviejo, and G. Escames, "Role of Melatonin in Cancer: Effect on Clock Genes," *International Journal of Molecular Sciences* 24, no. 3 (2023): 1919, <https://doi.org/10.3390/ijms24031919>.
70. T. Igarashi, H. Izumi, T. Uchiumi, et al., "Clock and ATF4 Transcription System Regulates Drug Resistance in Human Cancer Cell Lines," *Oncogene* 26, no. 33 (2007): 4749–4760, <https://doi.org/10.1038/sj.onc.1210289>.
71. P. Plaimée, N. Weerapreeyakul, S. Barusrux, and N. P. Johns, "Melatonin Potentiates Cisplatin-Induced Apoptosis and Cell Cycle Arrest in Human Lung Adenocarcinoma Cells," *Cell Proliferation* 48, no. 1 (2015): 67–77, <https://doi.org/10.1111/cpr.12158>.

72. U. Laothong, Y. Hiraku, S. Oikawa, K. Intuyod, M. Murata, and S. Pinlaor, "Melatonin Induces Apoptosis in Cholangiocarcinoma Cell Lines by Activating the Reactive Oxygen Species-Mediated Mitochondrial Pathway," *Oncology Reports* 33, no. 3 (2015): 1443–1449, <https://doi.org/10.3892/or.2015.3738>.
73. Y.-T. Chen, C.-C. Yang, P.-L. Shao, C.-R. Huang, and H.-K. Yip, "Melatonin-Mediated Downregulation of ZNF746 Suppresses Bladder Tumorigenesis Mainly Through Inhibiting the AKT-MMP-9 Signaling Pathway," *Journal of Pineal Research* 66, no. 1 (2019): e12536, <https://doi.org/10.1111/jpi.12536>.
74. G. Ji, W. Zhou, X. Li, J. Du, X. Li, and H. Hao, "Melatonin Inhibits Proliferation and Viability and Promotes Apoptosis in Colorectal Cancer Cells via Upregulation of the microRNA-34a/449a Cluster," *Molecular Medicine Reports* 23, no. 3 (2021): 187, <https://doi.org/10.3892/mmr.2021.11826>.
75. S. Lin, K. Hoffmann, C. Gao, M. Petrulionis, I. Herr, and P. Schemmer, "Melatonin Promotes Sorafenib-Induced Apoptosis Through Synergistic Activation of JNK/C-Jun Pathway in Human Hepatocellular Carcinoma," *Journal of Pineal Research* 62, no. 3 (2017): e12398, <https://doi.org/10.1111/jpi.12398>.
76. I. Bejarano, J. Espino, A. M. Marchena, et al., "Melatonin Enhances Hydrogen Peroxide-Induced Apoptosis in Human Promyelocytic Leukaemia HL-60 Cells," *Molecular and Cellular Biochemistry* 353, no. 1–2 (2011): 167–176, <https://doi.org/10.1007/s11010-011-0783-8>.
77. J. Perdomo, J. Cabrera, F. Estévez, J. Loro, R. J. Reiter, and J. Quintana, "Melatonin Induces Apoptosis Through a Caspase-Dependent but Reactive Oxygen Species-Independent Mechanism in Human Leukemia Molt-3 Cells," *Journal of Pineal Research* 55, no. 2 (2013): 195–206, <https://doi.org/10.1111/jpi.12062>.
78. M. Akbarzadeh, M. Nouri, M. V. Banekohal, et al., "Effects of Combination of Melatonin and Laser Irradiation on Ovarian Cancer Cells and Endothelial Lineage Viability," *Lasers in Medical Science* 31, no. 8 (2016): 1565–1572, <https://doi.org/10.1007/s10103-016-2016-6>.
79. W. Li, J. Wu, Z. Li, et al., "Melatonin Induces Cell Apoptosis in Mia PaCa-2 Cells via the Suppression of Nuclear Factor- κ B and Activation of ERK and JNK: A Novel Therapeutic Implication for Pancreatic Cancer," *Oncology Reports* 36, no. 5 (2016): 2861–2867, <https://doi.org/10.3892/or.2016.5100>.
80. C. Alonso-González, J. Menéndez-Menéndez, A. González-González, A. González, S. Cos, and C. Martínez-Campa, "Melatonin Enhances the Apoptotic Effects and Modulates the Changes in Gene Expression Induced by Docetaxel in MCF-7 Human Breast Cancer Cells," *International Journal of Oncology* 52, no. 2 (2018): 560–570, <https://doi.org/10.3892/ijo.2017.4213>.
81. R. Pariente, I. Bejarano, J. Espino, A. B. Rodríguez, and J. A. Pariente, "Participation of MT3 Melatonin Receptors in the Synergistic Effect of Melatonin on Cytotoxic and Apoptotic Actions Evoked by Chemotherapeutics," *Cancer Chemotherapy and Pharmacology* 80, no. 5 (2017): 985–998, <https://doi.org/10.1007/s00280-017-3441-3>.
82. S. A. Zakki, J. S. Muhammad, J.-L. Li, et al., "Melatonin Triggers the Anticancer Potential of Phenylarsine Oxide via Induction of Apoptosis Through ROS Generation and JNK Activation[†]," *Metallomics* 12, no. 3 (2020): 396–407, <https://doi.org/10.1039/c9mt00238c>.
83. N. Zhou, Z. X. Wei, and Z. X. Qi, "Inhibition of Autophagy Triggers Melatonin-Induced Apoptosis in Glioblastoma Cells," *BMC Neuroscience* Dec 23 20, no. 1 (2019): 63, <https://doi.org/10.1186/s12868-019-0545-1>.
84. W. J. Lee, L. C. Chen, J. H. Lin, et al., "Melatonin Promotes Neuroblastoma Cell Differentiation by Activating Hyaluronan Synthase 3-Induced Mitophagy," *Cancer Medicine* 8, no. 10 (2019): 4821–4835, <https://doi.org/10.1002/cam4.2389>.
85. R. R. Adler, A. K. Ng, and N. S. Rote, "Monoclonal Antiphosphatidylserine Antibody Inhibits Intercellular Fusion of the Choriocarcinoma Line, JAR," *Biology of Reproduction* 53, no. 4 (1995): 905–910, <https://doi.org/10.1095/biolreprod53.4.905>.
86. D. Lanoix, R. Ouellette, and C. Vaillancourt, "Expression of Melatoninergic Receptors in Human Placental Choriocarcinoma Cell Lines," *Human Reproduction* 21, no. 8 (2006): 1981–1989, <https://doi.org/10.1093/humrep/del120>.
87. S. S. Malhotra, P. Suman, and S. K. Gupta, "Alpha or Beta Human Chorionic Gonadotropin Knockdown Decrease BeWo Cell Fusion by Down-Regulating PKA and CREB Activation," *Scientific Reports* 5 (2015): 11210, <https://doi.org/10.1038/srep11210>.
88. M. Matsumoto, M. Minami, K. Takeda, Y. Sakao, and S. Akira, "Ectopic Expression of CHOP (GADD153) Induces Apoptosis in M1 Myeloblastic Leukemia Cells," *FEBS Letters* 395, no. 2–3 (1996): 143–147, [https://doi.org/10.1016/0014-5793\(96\)01016-z](https://doi.org/10.1016/0014-5793(96)01016-z).
89. X. Z. Wang, "Identification of Novel Stress-Induced Genes Downstream of CHOP," *The EMBO Journal* 17, no. 13 (1998): 3619–3630, <https://doi.org/10.1093/emboj/17.13.3619>.
90. M. Dowaidar, M. Gestin, C. P. Cerrato, et al., "Role of Autophagy in Cell-Penetrating Peptide Transfection Model," *Scientific Reports* 7, no. 1 (2017): 12635, <https://doi.org/10.1038/s41598-017-12747-z>.
91. S. B. Cullinan and J. A. Diehl, "PERK-Dependent Activation of Nrf2 Contributes to Redox Homeostasis and Cell Survival Following Endoplasmic Reticulum Stress," *Journal of Biological Chemistry* 279, no. 19 (2004): 20108–20117, <https://doi.org/10.1074/jbc.M314219200>.
92. S. B. Cullinan, D. Zhang, M. Hannink, E. Arvisais, R. J. Kaufman, and J. A. Diehl, "Nrf2 Is a Direct Perk Substrate and Effector of PERK-Dependent Cell Survival," *Molecular and Cellular Biology* 23, no. 20 (2003): 7198–7209, <https://doi.org/10.1128/mcb.23.20.7198-7209.2003>.
93. Y. Bu and J. A. Diehl, "PERK Integrates Oncogenic Signaling and Cell Survival During Cancer Development," *Journal of Cellular Physiology* 231, no. 10 (2016): 2088–2096, <https://doi.org/10.1002/jcp.25336>.
94. P. Wang, L. Han, M. Yu, et al., "The Prognostic Value of PERK in Cancer and Its Relationship With Immune Cell Infiltration," *Frontiers in Molecular Biosciences* 8 (2021): 648752, <https://doi.org/10.3389/fmolb.2021.648752>.
95. I. C. Salaroglio, E. Panada, E. Moiso, et al., "PERK Induces Resistance to Cell Death Elicited by Endoplasmic Reticulum Stress and Chemotherapy," *Molecular Cancer* 16, no. 1 (2017): 91, <https://doi.org/10.1186/s12943-017-0657-0>.
96. B. Guo, X. Xiong, S. Hasani, et al., "Downregulation of PHLPP Induced by Endoplasmic Reticulum Stress Promotes eIF2 α Phosphorylation and Chemoresistance in Colon Cancer," *Cell Death & Disease* 12, no. 11 (2021): 960, <https://doi.org/10.1038/s41419-021-04251-0>.
97. M. Tanabe, H. Izumi, T. Ise, et al., "Activating Transcription Factor 4 Increases the Cisplatin Resistance of Human Cancer Cell Lines," *Cancer Research* 63, no. 24 (2003): 8592–8595.
98. L. Fan, G. Sun, T. Ma, et al., "Melatonin Reverses Tunicamycin-Induced Endoplasmic Reticulum Stress in Human Hepatocellular Carcinoma Cells and Improves Cytotoxic Response to Doxorubicin by Increasing CHOP and Decreasing Survivin," *Journal of Pineal Research* 55, no. 2 (2013): 184–194, <https://doi.org/10.1111/jpi.12061>.
99. B. Zhou, Q. Lu, J. Liu, et al., "Melatonin Increases the Sensitivity of Hepatocellular Carcinoma to Sorafenib Through the PERK-ATF4-Beclin1 Pathway," *International Journal of Biological Sciences* 15, no. 9 (2019): 1905–1920, <https://doi.org/10.7150/ijbs.32550>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.
Supporting data.