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Original Articles

Exploiting Honokiol-induced ER stress CHOP activation inhibits the growth and metastasis of melanoma by suppressing the MITF and β-catenin pathways

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ABSTRACT

There is increasing global incidence of highly metastatic melanoma and therapeutic strategies like those focusing on the downstream beta-catenin/MITF axis of invading melanoma cells are urgently needed. Targeting endoplasmic reticulum (ER) stress can promote cancer cell death and inhibit epithelial mesenchymal transition (EMT) in metastatic tumors. This study aimed to determine if Honokiol could promote ER stress-dependent apoptosis and regulate metastatic melanoma. The therapeutic efficacy of Honokiol was assessed using the highly metastatic melanoma xenograft mouse model for peritoneal metastasis and evaluated by computed tomography imaging. The ER stress marker, Calpain-10, delineated a novel proteolytic cleavage enzyme, while CHOP/GADD153-regulated apoptosis was used for gene silencing to determine the role of the β-catenin/MITF axis in melanoma cells. The results showed that Honokiol effectively decreased peritoneal dissemination and organ metastasis via ER stress activation and EMT marker inhibition. Knockdown Calpain-10 or CHOP/GADD153 blocked all of the biological effects in Honokiol-induced β-catenin/MITF cleavage, ERSE or TCF/LEF luciferase activity, and β-catenin kinase activity. These findings suggest that Honokiol can significantly thwart the progression of highly metastatic melanoma using the β-catenin/MITF axis via prompt Calpain-10 and CHOP/GADD153 regulated cascades.

1. Introduction

Highly metastatic melanoma represents a major clinical challenge as the deadliest form of skin cancer. It is now the fifth most common cancer in the United States and its average incidence rate has increased by 2.6% annually in the last decade, although this increase also includes tumors with metastatic potential [1,2]. Despite emerging advances, multi-modality therapies have very limited therapeutic effects on rebellious metastatic melanoma. Alternative approaches and novel drugs with high efficiency and minimal toxicity need to be explored and developed. Recent reports show that the biphenolic natural product, Honokiol ($C_{18}H_{18}O_2$), has potential in anti-cancer therapy as its effects

Abbreviations: ER, endoplasmic reticulum; EMT, epithelial mesenchymal transition; MITF, Microphthalmia-associated transcription factor; TCFs, T-cell factors; ICD, immunogenic cell death; UPR, unfolded protein response; CHOP/GADD153, CCAAT-enhancer-binding protein homologous protein; PET/CT, Positron emission tomography/computed tomography

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are dependent on anti-neoplastic processes like the induction of apoptosis and vemurafenib-resistance and the inhibition of migratory potential in melanoma [3–6]. However, there is little report linking it to endoplasmic reticulum (ER) stress with the epithelial-mesenchymal transition (EMT) and metastasis in melanoma.

Epidemiologic studies and meta-analyses strongly suggest the preventive and curative potential of plants rich in polyphenol against cancer or chronic diseases [7–10]. Honokiol, extracted from the bark or seed cones of the *Magnolia* tree, may be an important potential chemopreventive agent as well as a therapeutic natural agent [8]. In recent reports, Honokiol confers immunogenicity by dictating calreticulin exposure, activating ER stress, and inhibiting EMT in immunogenic cell death (ICD) during cancer therapy [11]. Emerging data further suggest that Honokiol possesses anti-peritoneal dissemination and anti-metastatic properties [12–17]. It has activity against vemurafenib-resistant melanoma *in vivo* and increases both respiration and reactive oxygen generation [3]. It inhibits NADPH oxidase 1 activity and blocks the binding of cytosolic and membrane-bound proteins, thereby inhibiting the migratory potential of melanoma cells [6]. Honokiol can inhibit melanoma stem cells, partly by targeting the suppression of Notch-2 signaling [4]. Sengupta et al. present evidence to support crosstalk between activation of tumor suppressor LKB1, inhibition of oncogenic Stat3, and pluripotency factors in breast cancer. Honokiol has effective anti-cancer modulation of this axis *in vitro* and *in vivo* [18].

β-catenin-induced melanoma growth or metastasis requires the downstream target microphthalmia-associated transcription factor (MITF). Inhibiting this migration is dependent on MITF-M, a key transcription factor of the melanocyte lineage [19]. However, β-catenin may have conflicting roles in metastatic melanoma [20]. In the nucleus, stabilized β-catenin converts transcriptional repressors, T-cell factors (TCFs), into activators and regulates cell fate through gene expression [21–23]. In addition, MITF can interact with β-catenin to determine target gene expression or redirect β-catenin transcriptional activity away towards MITF-specific target promoters, thereby regulating melanocyte development and melanoma [21–23]. Lastly, β-catenin decreases during ciglitazonen treatment, indicating that activated PPARg inhibits the Wnt/β-catenin pathway [24].

The unfolded protein response (UPR) is a cellular stress response related to ER stress. The UPR is designed to mediate two conflicting outcomes: recovery and apoptosis. The CCAAT-enhancer-binding protein homologous protein (CHOP/GADD153) is induced by ER stress and mediates apoptosis, whereas CHOP is a multi-functional transcription factor in ER stress response [25]. Moreover, CHOP/GADD153 is an inhibitor of Wnt/TCF signals [26]. Until recently, reports have shown that apoptosis-related protein-2 triggers melanoma cell death by a mechanism that includes both ER stress and mitochondrial dysregulation, suggesting this control mechanism as an alternative approach for chemo-resistant metastatic melanoma [27]. Glucose availability controls the ATF4-mediated MITF suppression to drive melanoma cell growth [28] and inhibits the glycolytic pathway. Targeting ER stress and reducing basal autophagy may be suitable targets for novel combination therapies in a specific subgroup of metastatic melanoma. In such conditions, ER stress, acting as a molecular switch, dictates either cell survival or death [29].

This study aimed to determine if Honokiol can inhibit highly aggressive human melanoma cells and to define the molecular basis of this effect. This study also investigated the effect of Honokiol on the cellular localization of CHOP/GADD153 and the impact of CHOP/GADD153 on β-catenin proteins, with emphasis on MITF as a reciprocal effect of Wnt/β-catenin signaling.

2. Material and methods

2.1. Cell culture

Cell culture, viability assays, and drug treatment of melanoma cell lines B16F10 (Mouse), A375 and MeWo (Human malignant melanoma), and A2058 (Human metastatic melanoma) were obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute (BCRC/FIRDI, Taiwan). Cells were grown in DMEM or MEM medium that were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (complete medium) at 37 °C in humidified incubator with 5% CO2. Before drug treatment, the culture medium was changed to 0.5% fetal bovine serum medium. Honokiol was obtained from the Wako Chemical Company (Osaka, Japan).

2.2. Mouse xenograft model of animal peritoneal dissemination

The Committee for Animal Experiments of National Chung Hsing University approved all of the animal care and experimental procedures (Approval Document NCHU-100-26). Four-to six-week-old male BALB/c nude mice were purchased from the National Laboratory Animal Center (NLAC; Taipei, Taiwan). The mice were bred and maintained under specific pathogen-free conditions, given sterilized food and water ad libitum, and housed in a barrier facility with a 12-h light/dark cycle. In the peritoneal metastasis model, cultured B16F10 cells (1×10^5 cells) were inoculated into the peritoneal cavity of BALB/c nude mice.

Positron emission tomography/computed tomography (PET/CT) was conducted 12 days after the intra-peritoneal inoculation to confirm where substantial tumor has grown in the abdominal cavity. If the tumor was successfully inoculated, the mice were given an intra-peritoneal injection of Honokiol (1 mg/kg or 5 mg/kg, twice per week). Serial surveillance PET/CT imaging on day 21 after Honokiol treatment was used to detect peritoneal dissemination. The experimental mice were sacrificed under anesthesia and examined macroscopically for the presence of peritoneal metastasis. The tumors were excised, cut into blocks, fixed in 10% formalin, and embedded in paraffin blocks or snap-frozen in liquid nitrogen.

2.3. Positron emission tomography/computed tomography (PET/CT)

After at least 6 h of fasting, the mice were given a 7.4-MBq (0.2-mCi) dose of 18F-FDG orally before flushing with 1 ml water. The experimental mice were anesthetized with an isoflurane vaporizer before each scan. Experiments for small animal imaging were performed using a combined PET/CT scanner (Discovery ST; GE Medical Systems, Taichung, Taiwan). A multi-detector row helical CT scanner was used with the following technical parameters: CT scan type with a 0.5-s full helical scan; detector row configuration, 1661.25 mm; interval space, 2.75 mm; slice thickness, 1.25 mm; pitch, 1.75:1 (high-quality mode); speed, 17.5 mm per rotation; large field of view (FOV); voltage, 120 kVp; and current, 200 mA.

The technical specifications and parameters used for the PET portion included 10.0 min in each bed. The FOV for imaging reconstruction was 20 cm with PET resolution of about 4.5-mm full width at half maximum. The reconstructive parameters were type 3D iteration as 21 subsets and two iterations. To assess the quantification ability of the PET/CT scanner in small animal imaging, a region of interest was placed on smaller FOV *trans*-axial PET images to completely surround the areas of FDG uptake in the observed tissues while avoiding nearby tissues. The mean of each pixel's activity value within each region of interest was recorded and expressed as kBq/ml.

2.4. Western blot analysis and immuno-precipitation

Protein levels were analyzed by Western blot as previously described. Commercial antibodies against specific targeting protein were used and listed together with their sources. For immuno-precipitation, proteins (500 mg) were incubated with specific antibodies and immobilized onto protein A-Sepharose beads.

2.5. Calpain activity

Analysis of Calpain activity in total cell lysates was performed using Suc-Leu-Leu-Val-Tyr-AMC (Biomol). Quantitation of 7-amino-4-methylcoumarin (AMC) fluorescence was used to measure enzyme activity. The cells were prepared and treated on 24-well Corning/Costar plates. Before adding the inhibitors, the cells were loaded with 5 μM Calpain protease substrate and treated with Honokiol at 37 °C in a humidified 5% CO2 incubator. Proteolysis of the fluorescent probe was monitored using a fluorescent plate reading system (HTS-7000 Plus Series BioAssay, Perkin Elmer) with filter settings of 36 ± 20 nm for excitation and 46 ± 20 nm for emission.

2.6. TOP/FOP luciferase reporter assay

To assess the transcriptional activity of β-catenin, the TOP/FOP reporter system with the dual-luciferase kit (Dual-Glo™ Luciferase Assay System, Promega, Madison, WI, USA) was used. The cells were transiently transfected with constitutively active vector encoding Renilla luciferase and β-catenin-responsive firefly luciferase reporter plasmid TopFlash or the negative control FopFlash, and then harvested after 24 h in culture. Both the firefly and Renilla luciferase activities were measured in duplicate/triplicate according to the manufacturer's instructions. The firefly luciferase activity was normalized against the Renilla luciferase activity and the fold increase in TOPFlash activity compared to FOPFlash was reported.

2.7. Immuno-fluorescence confocal laser scanning microscopy

Cells were fixed in 4% paraformaldehyde for 30 min and then blocked by incubation in 1% bovine serum albumin in PBS. The primary antibodies were applied to the slides at a dilution of 1:500 and incubated at 4 °C overnight. The samples were treated with FITC-conjugated (Table 1) and then analyzed by immuno-fluorescence confocal laser scanning microscopy (see Tables 2 and 3).

2.8. In vitro kinase activity

Cell lysate was incubated in kinase assay buffer containing 10 μCi of [γ-^{32}P]ATP, 10 mM Tris-HCl (pH 7.4), 5 mM $MnCl_2$, 1 mM dithiothreitol, and 20 μM ATP for 20 min at 30 °C. Reactions were stopped by adding an equal volume of 2 × SDS-PAGE sample buffer and boiling for 5 min. Samples were then separated by 6% SDS-PAGE and transferred onto nitrocellulose membranes for exposure to x-ray film.

2.9. Colony-forming assay

Varying cell numbers (2×10^4/ml) were plated in 6-well plates and incubated at 37 °C in the absence or presence of treated as indicated for 10–14 day. Colonies were then stained with Crystal violet or Giemsa stain solution for 1 h at room temperature, and the stained colonies were counted using a ToupView Software.

2.10. Cell migration assay

Cells were seeded in 24-well plates in the absence or presence of transfected as indicated and cultured at 37 °C for 24 h. The cell monolayer was scratched using a 200 μL pipet tip, and the cells were cultured for an additional 8–12 h in the presence of Honokoil. Images were acquired at 0 and 12 h after the addition of Honokoil-treated. The width of the gap in the cell monolayer was measured using ToupView Software.

2.11. Web-based correlation

To verify the experimental study, the web tool and cancer databases were explored with clinical outcomes tailored to rapidly evaluate gene expression biomarkers and data by a valuable and comprehensive of SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp), PROGgeneV2 (http://watson.compbio.iupui.edu/chirayu/proggene/database/?url=proggene) as well as website: 'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)'.

2.12. Statistical analyses

All values given were presented as mean ± SEM. All analyses were performed using analysis of variance, followed by Fisher's least significant difference test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Honokiol blocked highly metastatic melanoma in vivo

Three groups of mice for metastatic dissemination experiments were

Table 1
Additional antibodies used in the study.

Name	Sp. (Clone number or code number)		In the work usage	Vendor
Primary antibody				
IRE1α	sc-20790	rabbit polyclonal IgG	WB	Santa Cruz Biotechnology
GADD153	sc-7351	mouse monoclonal IgG1	WB, IP	Santa Cruz Biotechnology
GAPDH	sc-8035	mouse monoclonal IgM	WB	Santa Cruz Biotechnology
Calpain 1	sc-7531	goat polyclonal IgG	WB	Santa Cruz Biotechnology
Calpain 2	sc-7533	goat polyclonal IgG	WB	Santa Cruz Biotechnology
Calpain 10	sc-48454	goat polyclonal IgG	WB,IP	Santa Cruz Biotechnology
GRP78	sc-13968	rabbit polyclonal IgG	WB	Santa Cruz Biotechnology
GRP94	sc-11402	rabbit polyclonal IgG	WB	Santa Cruz Biotechnology
p-eIF2α(Ser51)	9721	polyclonal antibodies	WB	Cell Signaling
Snail	sc-28199	rabbit polyclonal IgG	WB	Santa Cruz Biotechnology
Vimentin	sc-7557	goat polyclonal IgG	WB	Santa Cruz Biotechnology
Slug	sc-10437	goat polyclonal IgG	WB	Santa Cruz Biotechnology
Twist	sc-6269	goat polyclonal IgG	WB	Santa Cruz Biotechnology
p- E-cadherin (Ser838 + Ser840)	ab76319	monoclonal antibody	WB	Abcam
p-Tpl2	4491	Polyclonal antibodies	WB	Cell Signaling
Cytokeritin18	sc-32329	mouse monoclonal IgG1	WB	Santa Cruz Biotechnology
ZO-1	sc-10804	rabbit polyclonal IgG	WB	Santa Cruz Biotechnology
β-catenin	sc-7963	mouse monoclonal IgG	WB	Santa Cruz Biotechnology
MITF	ab12039	Mouse monoclonal	WB, IP, IF	Abcam
Cdk2	sc-163	rabbit polyclonal IgG	WB	Santa Cruz Biotechnology

Table 2
Additional antibodies used in the study.

Name	Sp. (Clone number or code number)	In the work usage	Vendor
Secondary antibody			
Fluorescein-Labeled Antibody To Mouse IgG (H + L)	172–1806	IF	Kirkegaard&Perry Laboratories, Inc
Peroxidase-conjugated affinipure goat anti-mouse IgG(H + L)	115-035-003	WB	Jackson IrnrnunoReserch Laboratories, Inc
Peroxidase-conjugated affinipure goat anti-rabbit IgG(H + L)	111-035-003	WB	Jackson IrnrnunoReserch Laboratories, Inc
Donkey anti-goat HRP	Sc-2020	WB	Kirkegaard & Perry Laboratories, Inc

Abbreviations:WB: Western blot; IF: immunofluorescence.

investigated (Fig. 1A). The maximum intensity projection was generated from typical representative control group mice (Fig. 1B–a, b, f, and g) and was effectively reversed by intra-peritoneal Honokiol treatment 1 mg/kg (Fig. 1B–c, d, and e) or 5 mg/kg (Fig. 1B–h and i). The non-invasive glucose tracer [^{18}F]-FDG uptakes in the peritoneal metastatic melanoma of the control group were much higher than those in the Honokiol-therapy group (Fig. 1B).

Many metastatic nodules were found in the peritoneal cavity (mesentery) of the control mice inoculated with B16F10 melanoma cells (Fig. 2A, upper panel). In contrast, peritoneal tumor nodules were markedly reduced or with only sporadic remnants in Honokiol-treated mice (Fig. 2A, middle panel, 1 mg/kg; lower panel, 5 mg/kg). Comparing the microscopic representations for peritoneal dissemination, ascites, and organ metastasis (i.e., lung, liver, gastro-intestinal tract, spleen, and stomach), the quantification of nodules in pathologic features per field demonstrated that Honokiol efficiently inhibited highly metastatic melanoma formation (Fig. 2Ba-g).

3.2. Honokiol prompted ER stress and Calpain-10 activity and delayed EMT markers

Honokiol significantly increased ER stress indicator proteins (p-elf2α, IRE1α, CHOP/GADD153, and Calpain-10) and decreased Tpl2 phosphorylation (Fig. 3A). There was no difference in Calpain-1, Calpain-2, GRP78, and GRP94. For EMT markers, there were decreased vimentin, β-catenin, and MITF levels, and increased p-Eadherin, but no difference in snail, slug, twist, ZO-1, and CK-18. Honokiol also led to a 1.88- to 3.2-fold increase in ER stress activity compared to controls. Similar results were observed with Tunicamycin. In contrast, Calpain inhibitors Nacetyl-leu-leu-norleucinal (ALLN) and N-acetyl-leu-leu-methioninal (ALLM) efficiently abolished Honokiol-induced ERSE reporter activation. Gene silencing of Calpain-10, but not of Calpain-1 and Calpain-2, also prevented the inducing effects (Fig. 3B). Honokiol increased Calpain activity in a time-dependent manner starting at 15 min, peaking at 30 min, and then dropping to a lower level at 4 h (Fig. 3C). Increased Calpain activity in response to Honokiol at 30 min was blocked by specific knockdown Calpain-10 (si-Calpain-10) (Fig. 3D). Calpain-1 and Calpain-2 had no effect.

3.3. Honokiol enhanced Calpain10 and MITF-m interaction and caused MITF-m cleavage

Honokiol markedly reduced MITF-M at 8–12 h, which was reversed by ALLN or ALLM in a dose-dependent manner (Fig. 4A). β-catenin was decreased starting at 18–24 h (data not shown). Knockdown by si-Calpain-10, but not si-Calpain-1 or si-Calpain-2, significantly restored MITF production in MeWo or B16F10 melanoma cells (Fig. 4B). In confocal fluorescence microscopy, Honokiol blocked MITF accumulation in the nucleus of A2058 and reduced cytosolic MITF intensity (Fig. 4C). Immuno-precipitation revealed that Calpain10 interplayed with MITF after 2–4 h of Honokiol exposure (Fig. 4D). These demonstrated that Honokiol-induced Calpain-10 activation was crucial for MITF and β-catenin expression.

Table 3
Additional shRNA used in the study.

Name shRNA	Sp. (Clone number or code number)		In the work usage	Vendor
Vimentin	TRCN000089832	NM_011701	TF	National RNAi Core Facility Platform
vimentin	TRCN000089828	NM_011701	TF	National RNAi Core Facility Platform
Calpain 10 (m)	TRCN0000030705	NM_011796	TF	National RNAi Core Facility Platform
Calpanin 10 (m)	TRCN0000030706	NM_011796	TF	National RNAi Core Facility Platform
Calpain 10 (h)	TRCN0000051928	NM_021251	TF	National RNAi Core Facility Platform
Calpanin 10 (h)	TRCN0000234884	NM_021251	TF	National RNAi Core Facility Platform
CHOP/GADD153 (m)	TRCN0000311413	NM_007837	TF	National RNAi Core Facility Platform
CHOP/GADD153 3 (m)	TRCN0000324349	NM_007837	TF	National RNAi Core Facility Platform
CHOP/GADD153 (h)	TRCN0000364328	NM_004083	TF	National RNAi Core Facility Platform
CHOP/GADD153 (h)	TRCN0000364393	NM_004083	TF	National RNAi Core Facility Platform
Name siRNA	Sp.(Clone number or code number)		In the work usage	Vendor
Calpain 1 (h)	sc-29885		TF	Santa Cruz Biotechnology
Calpain 1 (m)	sc-29886		TF	Santa Cruz Biotechnology
Calpain 2 (h)	sc-41459		TF	Santa Cruz Biotechnology
Calpain 2 (m)	sc-41460		TF	Santa Cruz Biotechnology
Calpain 10 (h)	sc-60318		TF	Santa Cruz Biotechnology
Calpain 10 (m)	sc-60319		TF	Santa Cruz Biotechnology
MITF (h)	sc-35934		TF	Santa Cruz Biotechnology
MITF ((m)	sc-35934		TF	Santa Cruz Biotechnology
GADD153 (h)	sc-35437		TF	Santa Cruz Biotechnology
GADD153 (m)	sc-35438		TF	Santa Cruz Biotechnology
β-catenin (h)	sc-29209		TF	Santa Cruz Biotechnology
β-catenin (h)	sc-29210		TF	Santa Cruz Biotechnology

Abbreviations:WB: Western blot; IF: immunofluorescence; IHC(P):immunohistochemistry(paraffin); TF: transfection.

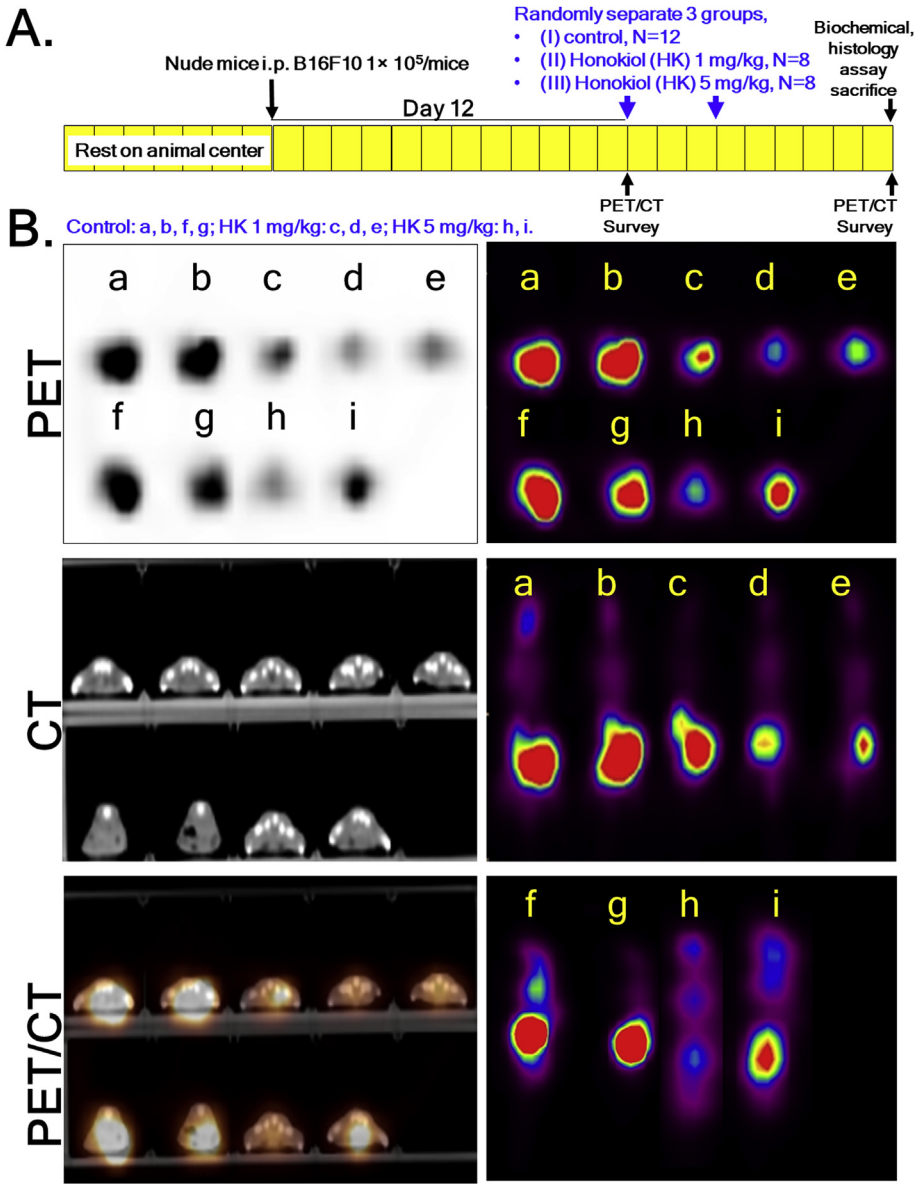

Fig. 1. Honokiol blocked highly metastatic dissemination of melanoma in vivo. (A) Schematic representation of the peritoneal metastasis animal model with and without Honokiol. Three groups of mice were investigated and listed. (B) PET/CT surveillance of the metastatic conditions of the B16eF10 melanoma cells after inoculation and after Honokiol treatment. The B16eF10 murine melanoma cells were inoculated onto the mice on day 12 after Honokiol therapy. Control group: a, b, f, g (n = 12); HK-treated group 1 mg/kg: c, d, e (n = 8); HK-treated group 5 mg/kg: h, I (n = 8). The axial cut was presented at the PET (upper left panel), CT (middle left panel), and PET/CT (lower left and upper right panels). The coronal cut was presented at the middle right and lower right panels.

3.4. Honokiol-induced CHOP/GADD153 regulated β-catenin activation

Honokiol increased the CHOP/GADD153 expression in metastatic melanoma cells (Fig. 3A), while Honokiol with Tunicamycin markedly inhibited reporter activity in A2058, B16F10 and MeWo cell lines (Fig. 5A). Small-interfering RNA (si-RNA)-mediated gene silencing revealed that si-Calpain-10 reversed luciferase activity, but si-Calpain-1 and si-Calpain-2 had no effect. si-CHOP/GADD153 restored Wnt/β-catenin activity with Topflash reporters. Examining phosphorylation of canonical Wnt pathway is, Honokiol or Tunicamycin induced p-β-catenin, a blocking peptide that restrained β-catenin (Fig. 5B left panel), while si-CHOP/GADD153 significantly prohibited p-β-catenin. In immuno-precipitation, Honokiol enhanced CHOP/GADD153 and MITF interplay (Fig. 5C). Knockdown of β-catenin also efficiently blocked the related target gene MITF and CDK2 expressions (Fig. 5D). These indicated that Honokiol efficiently induced CHOP/GADD153 to regulate MITF and β-catenin.

3.5. Honokiol induced ER stress and reduced MITF, β-catenin, and CDK2 expressions in animal melanoma tissues

In animal melanoma tissues, Honokiol significantly increased the ER stress marker p-elf2a, Calpain-10, and CHOP/GADD153. Vimentin was decreased but E-cadherin was elevated (Fig. 6A left panel). Moreover, MITF, β-catenin, and CDK2 were also effectively down-regulated in the Honokiol-treated group (Fig. 6A right panel).

The profile for animal tissues was similar in histologic imaging. In H &E staining, there was decreased melanin deposition by Honokiol administration. In fluorescence or IHC staining, there were increased CHOP/GADD153 and Calpain-10 intensities, and decreased MITF, β-catenin, and CDK2 expressions. These were consistent with the above-mentioned results and proof in the animal study (Fig. 6B). Several indicators were significantly suppressed by Honokiol, with a model of working hypothesis. All of these data demonstrated that CHOP/GADD153 activation, MITF and β-catenin suppression, β-catenin phosphorylation, or exposure to Honokiol-induced Calpain-10 activity could all reduce tumor growth and peritoneal metastasis in tumor tissues.

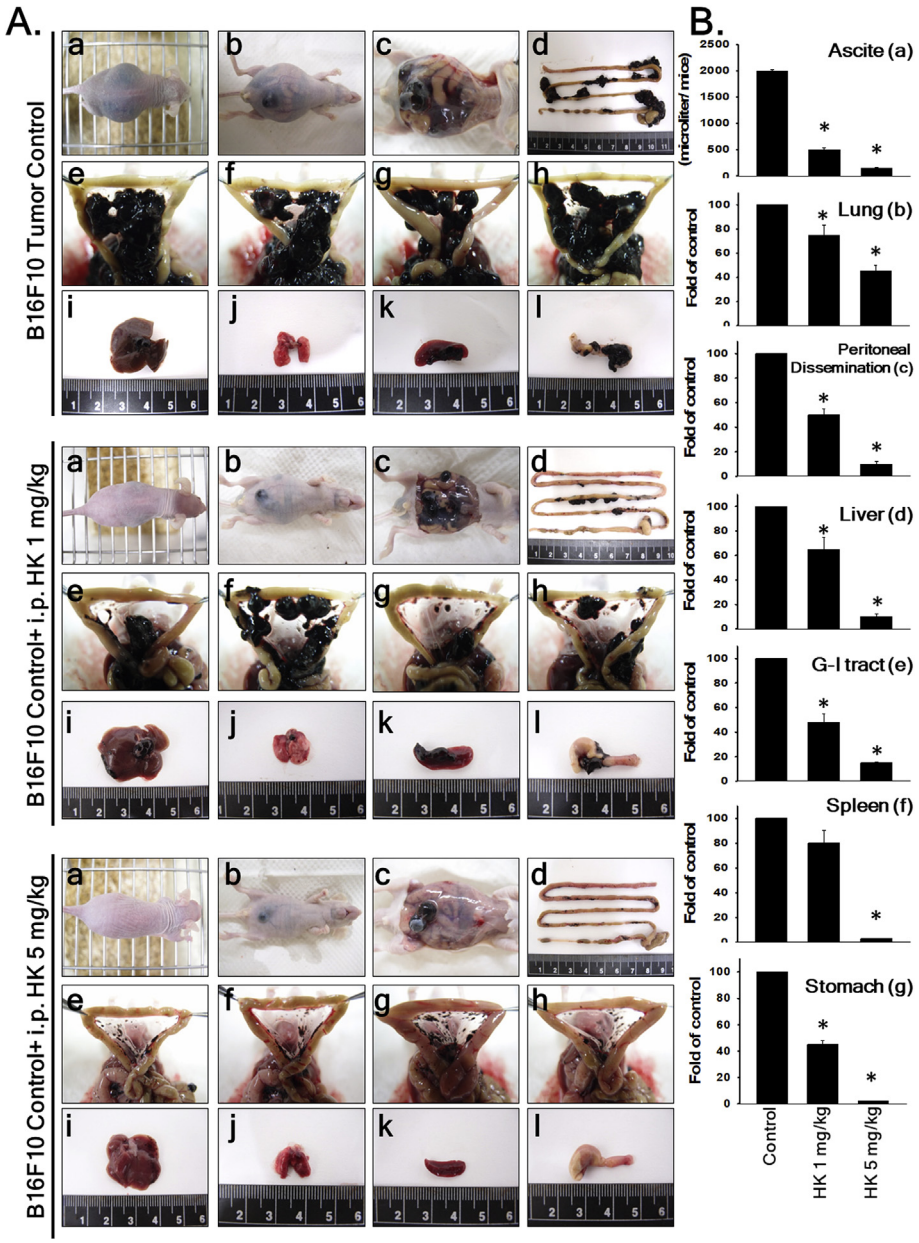

Fig. 2. Macroscopic imaging of Honokiol on peritoneal dissemination and organ metastasis of melanoma cancer cells. (A) A representative image showed the peritoneal dissemination in the control and Honokiol-treated animals. On physical exam, the experimental model had a large metastatic mass in the internal organ and a distended abdomen due to ascites. Distant metastases were found in the lungs, peritoneum, liver, gastrointestinal tract, and spleen, as indicated. Both ascites and organ metastasis were markedly reduced in Honokiol-treated mice. (B) Quantification of the volume or number of nodules was estimated by weighing or counting (right panel). Data were presented as mean ± SEM (n = 8 to 12).

3.6. High expressions of MITF, β-catenin, and CDK2 decreased the overall survival probability

By web-based correlation, there was a significantly positive correlation between the high expressions of MITF, β-catenin, and CDK2 in 44 patients with metastatic melanoma, as annotated in the Bogunovic Skin Survival GSE19234 dataset or by TCGA (Figs. 7A and 8A, respectively). Similar patterns were also found in 16 metastasis of melanoma represented in the Skin Cutaneous Melanoma of TCGA dataset (Supplementary Figure.1A upper panel). In an animal study, the therapeutic effect of Honokiol-induced Calpain-10 (CAPN10) and CHOP/GADD153 (DDIT3) in a cohort divided at the median expression was also confirmed in the PROGgeneV2, GSE19234 dataset enhancements on the existing PROGgeneV2 database for CAPN10 (Calpain-10) and DDIT3 (CHOP/GADD153). There were several modifications to enhance the survival analysis capability of the tool (Fig. 7B). The Skin Cutaneous Melanoma of the TCGA dataset of cohort divided at the 75th percentile of gene expression was likewise confirmed in the PROGgeneV2 (Supplementary Figure. 1B lower panel). Moreover, individual CAPN10 (Calpain-10) and DDIT3 (CHOP/GADD153) were analyzed by Kaplan Meier in the R2: Genomics Analysis and Visualization Platform in Tumor Melanoma Metastatic –Bhardwaj-44-fRMA-u133p2 dataset (Fig. 7B). The same of results were shown in the Bhardwaj-44-MAS5.0-u133p2 dataset for CAPN10 (Calpain-10) (Supplementary Figure. 2A lower panel) and DDIT3 (CHOP/GADD153) (Supplementary Figure. 2B lower panel). Taken together, such information suggested that MITF, β-catenin, and CDK2 were constitutively active in human metastatic melanoma. The pharmacologic induction of Calpain-10 and CHOP/GADD153 expressions were useful surrogate markers for treatment activity.

4. Discussion and conclusions

Melanoma is an invasive cancer with increasing incidence and a lifetime risk of becoming intrinsically resistant to chemotherapy and apoptosis. The ER stress-induced cells apoptosis-based molecular therapies are desirable, but the identification of relevant gene target(s) is a major clinical challenge. The salient features of the data presented

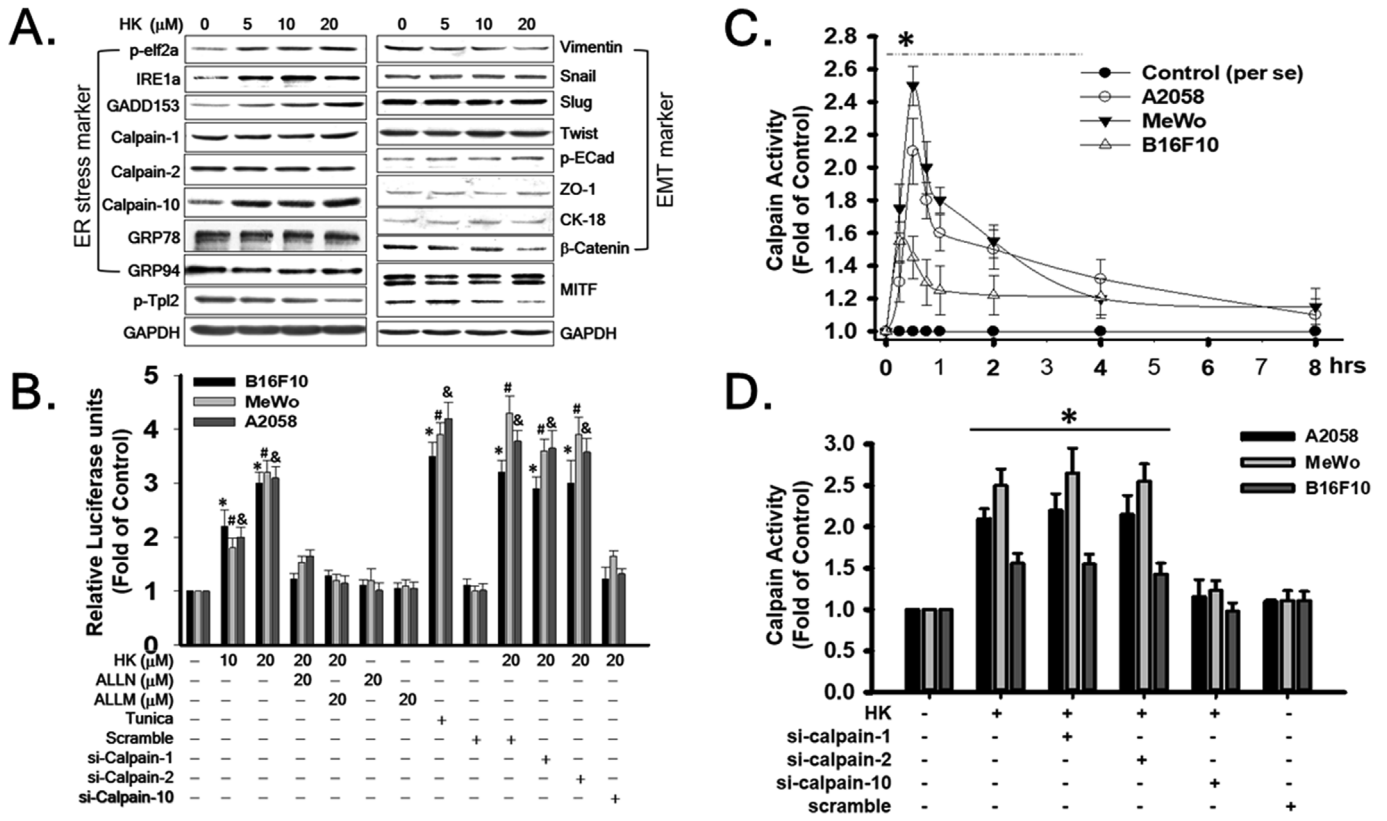

Fig. 3. Honokiol prompted ER stress and Calpain-10 activity, and delayed EMT markers in melanoma cells. (A) Honokiol treatment for 24 h in B16F10 melanoma cells induced ER stress-related molecules in a dose-dependent manner (left panel; control (0), 5, 10, 20 μM). The ER stress markers (p-elf2α, IRE1α, GADD153, Calpain-1, Calpain-2, Calpain-10, GRP78, GRP94 and p-Tpl2) were evaluated by Western blotting. Exposure to Honokiol of B16F10 melanoma cells induced EMT-related molecules in a dose-dependent manner (right panel). The EMT markers (Vimentin, Snail, Slug, Twist, p-Ecad, ZO-1, CK-18, β-Catenin, and MITF) were also examined by Western blotting. (B) ER stress activity by ERSE reporter detection (Cignal ERSE Reporter Assay Kit) was a measure of ER stress signaling. Melanoma cells (B16F10 MeWo and, A2058) were transfected with ERSE reporter, negative control, and positive control. Promoter activity values were expressed as arbitrary units using a Renilla reporter for internal normalization. The experiments were done in triplicate, with standard deviation as indicated. Data shown were representative of at least three independent experiments. (C) Calpain activity was measured by Suc-LLVY-AMC in indicated melanoma cells (A2058, MeWo, and B16F10) as an indicator of time course responses to Honokiol treatment. Data were expressed in terms of fold in control conditions. (D) Honokiol (20 μM) increased Calpain activity at 30 min treatment. Calpain inhibitors ALLN, ALLM, and Z-Leu-Leu-CHO (15 and 20 μM, data not shown) and gene silencing of Calpain-10 significantly inhibited Honokiol-induced Calpain activity. Data were presented as mean ± SEM (n = 4).

here is that Honokiol triggers apoptosis in metastatic melanoma through CHOP/β-Catenin and Calpain-10/MITF activation. While the increased efficacy of ER stress-induced apoptosis may offer considerable benefit in melanoma therapy, its potential effect on peritoneal dissemination have not well been identified. Emerging evidence indicates that ER stress activation can, in some circumstances, promote cell death. The stimulation of cytotoxic ER stress represents an alternative approach to ER stress modulation. The current study reveals that Honokiol exerts its anti-tumor effect on melanoma cells via activation of ER stress, subsequent apoptosis, and blocking of EMT characteristics. Thus, Honokiol may have clinical benefits for metastatic melanoma. Schematic of proposed mechanism for the role of Honokiol and blocked EMT and metastatic dissemination in melanoma (Fig. 8).

The molecular mechanisms connecting ER stress to cell death remain poorly understood and reports describing multiple molecular interactions involving ER stress are mostly equivocal. The pure compound Honokiol exerts its effect via ER-resident Caspase-12, whose activation has been proposed to be mediated by Calpain and Caspase processing, specific Calpain-10-dependent inhibition of MITF signaling, and ER stress-mediated apoptosis synchronously. Emerging reports demonstrate that most genes mediating pro-survival and cyto-protective functions have been identified as direct targets of microphthalmia-associated transcription factor (MITF), a melanocyte-specific modulator that is also a lineage addiction oncogene in metastatic melanoma [30,31]. The MITF has been identified as a key switch between cell survival and apoptosis during stress responses and its participation in melanoma response to α-Mangostin and Sorafenib may direct the cellular process towards apoptosis in the context of an NRAS mutant melanoma [32]. Moreover, MITF plays a dual role in the Wnt signaling pathway: it is a downstream target and is a nuclear mediator of Wnt signals in melanocytes [33]. Determining how to deregulate or inactivate the Wnt/β-catenin pathway, alone or with other pathways, in order to orchestrate the induction of target genes involved in a diverse range of activities represents a major challenge in research. Regulatory mechanisms include epigenetic and micro-environmental signals blocked by pharmacologic inhibition [34]. Various cancers in clinical therapy broadly utilize numerous transcription factors and signaling cascades in the reciprocal regulation of MITF expression and/or activity, including the Wnt/β-catenin pathway. In contrast, others, like BRAF(V600E)/ERK1/2, are more specific to melanoma [34]. Micro-environmental stress can block the autocrine and paracrine signaling of the Wnt/β-catenin pathway and negatively affect tumor growth. This, in turn, is induced by ER stress that inhibits normal Wnt protein processing and secretion in hypoxia condition [35]. Moreover, one of the markers of the ER stress-mediated apoptosis pathway is C/EBP homologous protein (CHOP), also known as growth arrest- and DNA damage-inducible gene 153 (GADD153). This has been shown to be an inhibitor of Wnt/TCF signals [36].

In the present work, Honokiol, as an inhibitor of the canonical Wnt pathway in metastatic melanoma cells, induces CHOP/GADD153. It

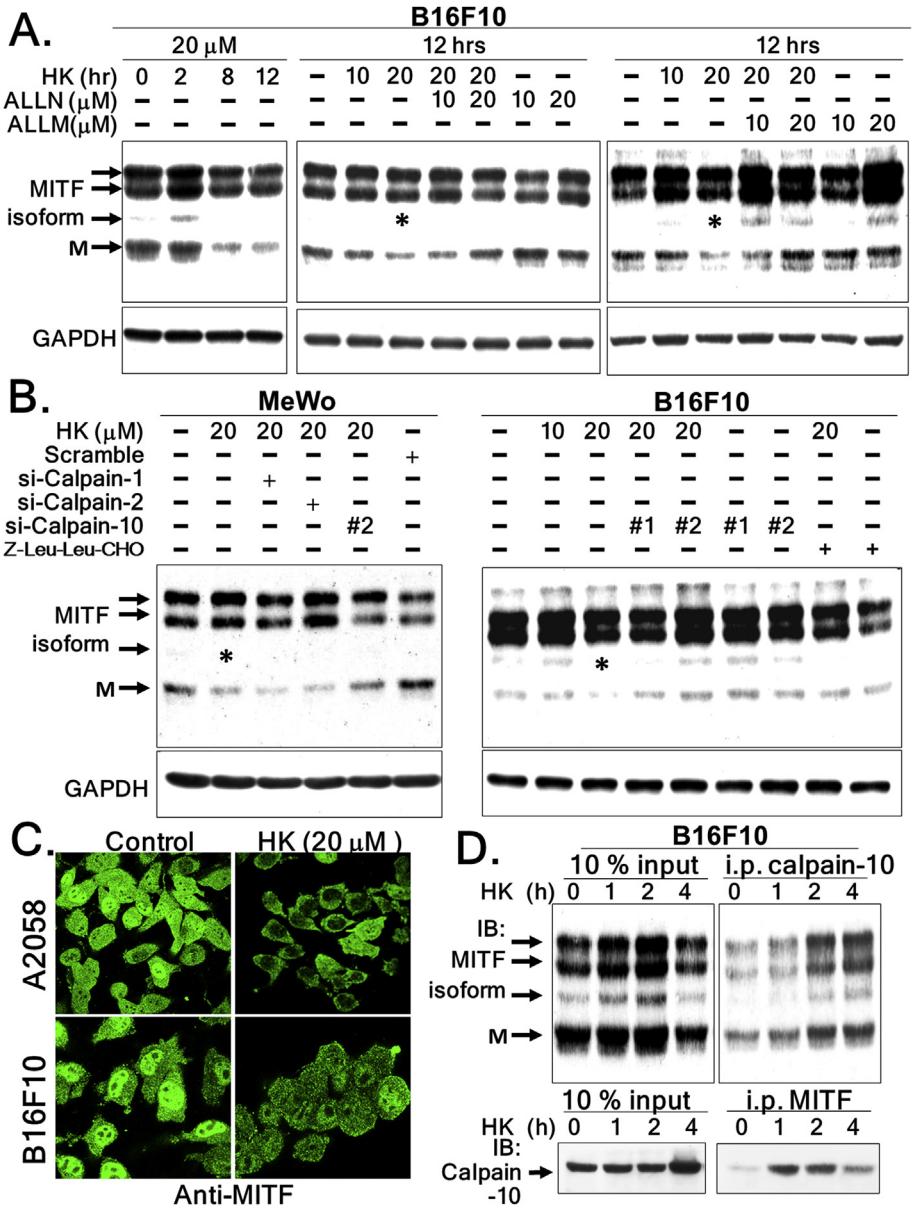

Fig. 4. Honokiol enhanced Calpain10 and MITF-m interaction, causing MITF cleavage. To determine the effects of (A) pre-treatment with Calpain inhibitors and (B) gene silencing of Calpain-1, Calpain-2, and Calpain-10, the B16eF10 melanoma cells exposed to Honokiol for 24 h were evaluated for MITF protein expression. (C) Primary antibodies for MITF were applied to the melanoma cells (A2058 and B16F10), followed by secondary antibodies coupled with conjugated FITC. Trans-localization of labeled antigens was detected through imaging. (D) Interaction of Calpain-10 and MITF were detected in B16eF10 melanoma cells. Immuno-precipitated proteins were collected and subjected to SDS-PAGE and immunoblotting with anti-Calpain10 and MITF antibodies. Results shown were representative of at least three independent experiments.

delineates a novel mechanism for this growth arrest gene. While CHOP/GADD153 interacts with MITF and β-catenin, silencing it inhibits the phosphorylation of β-catenin, triggering the n-catenin recruitment of ubiquitin E3 β-TrCP (β-transducin repeats-containing proteins). This causes its ubiquitination and proteasomal degradation that result in low levels of cytoplasmic β-catenin [26,37]. Future studies should clarify the physiologic function of CHOP/GADD153 during angiogenesis and tumorigenesis, and its role in blocking patho-physiologic states such as ectopic Wnt signaling, tumor formation, and metastasis. Honokiol has recently been shown to confer immunogenicity by dictating calreticulin exposure, activating ER stress, and inhibiting epithelial-to-mesenchymal transition [11]. *In vivo*, Honokiol DCA acts against LM36, a BRAF mutant melanoma, and LM36R, a vemurafenib-resistant clone of LM36. Honokiol DCA has been demonstrated to act in vemurafenib-resistant melanomas to increase both respiration and reactive oxygen generation, leading to activity against aggressive melanoma *in vivo* [3]. Honokiol analogs may be useful in treating highly resistant subsets of melanoma. Moreover, Honokiol administration rapidly reduces mitochondrial respiration by broadly inhibiting ETC complexes I, II, and V. This leads to decreased ATP levels. The subsequent energy crisis evokes two cellular responses involving cyclin-dependent kinases (CDKs) and promotes a pro-survival role of mitochondrial function in melanoma cells after oncogenic MAPK inhibition [38]. Honokiol also inhibits melanoma stem cells by targeting notch signaling, a pathway involved in stem cell self-renewal [4]. It causes significant accumulation of the cytosolic p47(phox) protein and decreased levels of the membrane-bound p22(phox) protein, thereby blocking their interaction and inhibiting Nox1 activation. *In vivo*, bioluminescence imaging data reveals that oral administration of Honokiol inhibits the migration/extravasation and growth of intravenously injected melanoma cells in internal body organs (i.e., liver, lungs, and kidneys) of nude mice. This has been associated with an inhibitory effect on Nox1 activity in such organs/tissues [6]. However, none of these results involving the targeting of ER stress has been correlated with EMT and melanoma invasion or metastasis, which may be a strong rationale for the development of new therapies for aggressive melanoma. To date, little is known about the protein expression or cellular function of Calpain-10 or the cellular and molecular mechanisms related to ER stress in the development and progression of aggressive metastatic melanoma. In the present work, Honokiol activates Calpain-10 activity and protein expression, but not traditional m- (Calpain-1) or μ-Calpains (Calpain-2). Transient transfection with siRNA-Calpain-10 effectively reverses

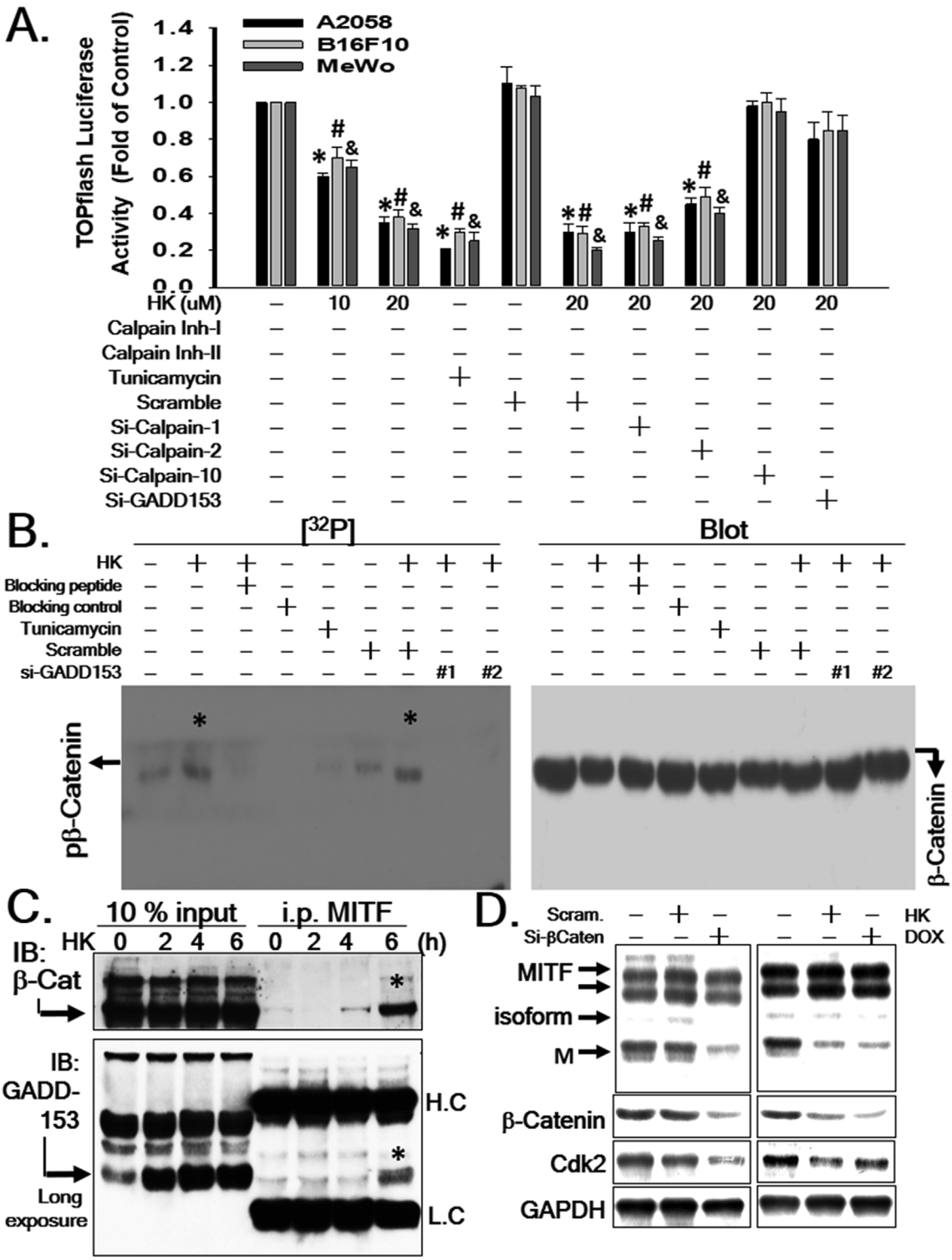

Fig. 5. Honokiol-induced GADD153/CHOP activation was involved in β-catenin phosphorylation and protein degradation. (A) The TCF/LEF reporter assay monitored the activity of Wnt signal transduction pathways in cultured melanoma cells A2058, B16F10 and MeWo after transfection with siCalpain-1, siCalpain-2, siCalpain-10, and siCHOP/GADD153, and exposure to Honokiol after 20 h. Dual luciferase reporter assays elucidated the transcriptional activity of nuclear β-catenin. Increased firefly luciferase activity was detected in cells transfected with TOPFlash compared to cells transfected with FOPFlash. Tunicamycin had a synchronous effect in the assay. (B) Kinase activity for β-catenin phosphorylation. (C) Interaction of MITF, β-catenin, and CHOP/GADD153 was detected by immuno-precipitation (IP). (D) After gene silencing of β-catenin following Honokiol or Doxorubicin (DOX) treatment, the cells were further subjected to SDS-PAGE and immuno-blotting with anti-MITF, β-catenin, and Cdk2 antibodies. The results represented three independent experiments.

Honokiol-induced ER stress, Calpain activity, and MITF, as well as the down regulation of canonical Wnt signaling activity. In addition, siRNA-CHOP/GADD153 can reverse the decreased TOPFlash Luciferase activity caused by Honokiol and Tunicamycin in melanoma. Moreover, the profile for biological effects in particular inhibitors or gene silencing was further proved such as soft agar colony formation assay (Supplementary Figure. 3) and cell migration assay (Supplementary Figure. 4). These results provide evidence that Calpain-10 activation

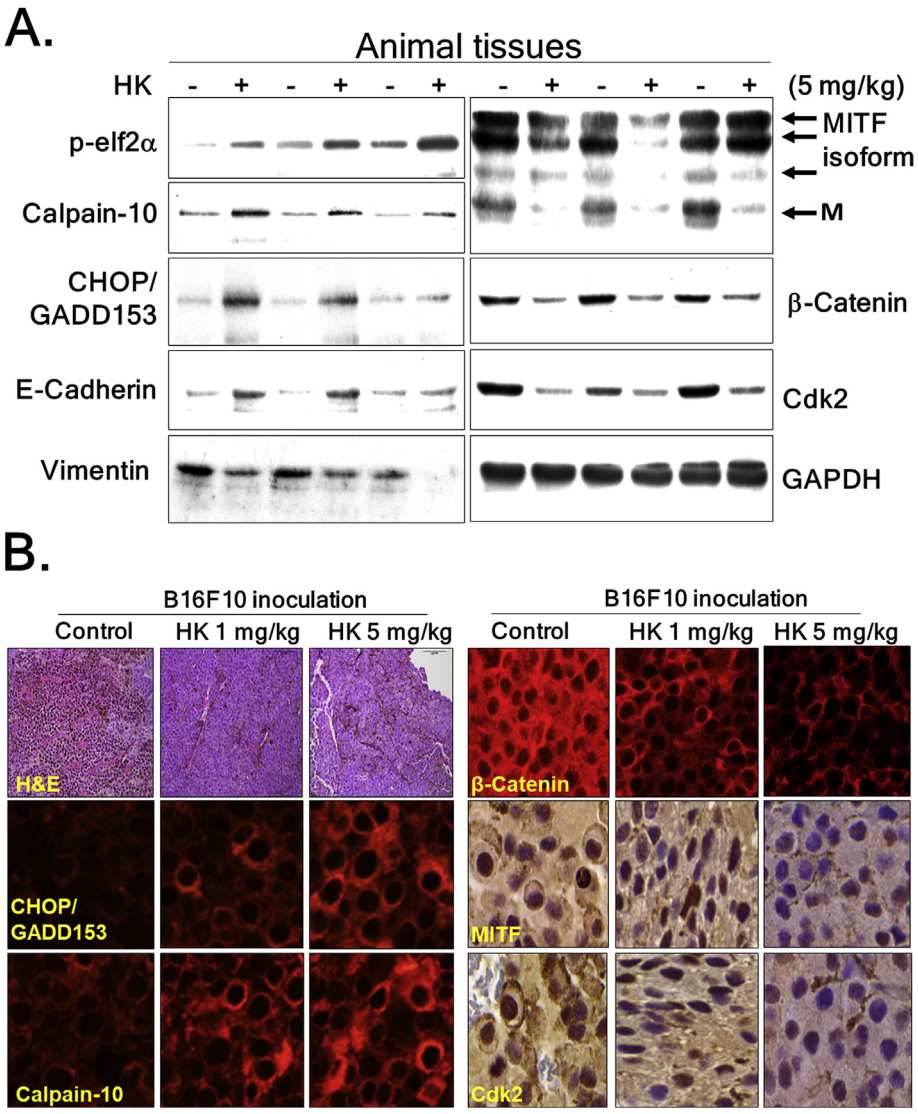

Fig. 6. Honokiol induced ER stress and reduced MITF, β-catenin, and Cdk2 expressions in animal melanoma tissues. (A) Animal tissues were targeting ER stress markers p-elf2a, Calpain-10, and CHOP/GADD153. In addition, vimentin, E-cadherin, MITF, β-catenin, and Cdk2 were also elevated. (B) H& E staining and immunohistochemistry of the tumor stained with anti-CHOP/GADD153 and Calpain-10 (left panel) and anti-β-catenin, MITF, and Cdk2 (right panel).

that dampens EMT is induced in melanoma cells under ER stress, which in turn can be provoked by Honokiol treatment. Hence, Calpain-10 activation may be another atypical ER stress marker.

Emerging evidence reveals that the development and physiologic differentiation of melanocytes in aggressive metastatic melanoma share common signaling pathways with many other neoplasms. These include the PI3 kinase and ERK pathways [34,39]. In addition, MITF controls the expression of melanogenic enzymes (TYR, TYRP1, and DCT) and regulates gene expression in all steps of melanocyte differentiation, including dendricity (DIA), melanosome biogenesis (SILVER, OA1), and melanosome transport (RAB27a) [40]. Others report that MITF expression is maintained in > 85% of melanoma and that MITF gene amplification has been shown in > 20% of aggressive metastatic melanomas. Further, MITF is indicated in controlling gene expressions in cell survival (BCL2, BIRC7), cell cycle control, and cellular processes [41–43]. On the other hand, MITF knockdown prevents melanoma cells in G0/G1 and evokes senescence [40]. These provide compelling evidence for a crucial role of MITF in melanoma development and are useful in pharmaco-therapeutics. Interestingly, MITF, a target for β-catenin, has a role in melanoblast proliferation and differentiation. The Wnt/β-catenin signaling pathway has been implicated in melanocyte development and malignant melanoma formation. However, β-catenin-induced melanoma growth requires the downstream targeting of MITF, suggesting that the many signaling pathways that converge on MITF may have additional cues involved in the genesis of melanomas similar to that of the Wnt pathway [20]. Functional studies revealed critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. Melanoma with low MITF expression have low levels of CDK2 and are particular susceptible to G1 arrest induced by siRNA-mediated CDK2 depletion [44]. In addition, the expressions of MITF and CDK2 are tightly correlated in primary melanoma specimens and predict susceptibility to the CDK2 inhibitor roscovitine [45]. This report is consistent with those findings. In the study, Honokoil reduced MITF and CDK2 expression. And entirely compatible in overall survival probability in melanoma. Moreover, previous study demonstrated that CDK2 phosphorylation on T160 increases during S phase and G2, when CDK2 is most active. Phosphorylation on the inhibitory sites T14 and Y15 is also maximal during S phase and G2 [46]. These probably functional specific phosphorylated form of the kinase active site need to be further investigated.

The CAAT/enhancer binding protein (C/EBP) homologous protein, GADD153, is a stress response protein and growth arrest gene that can

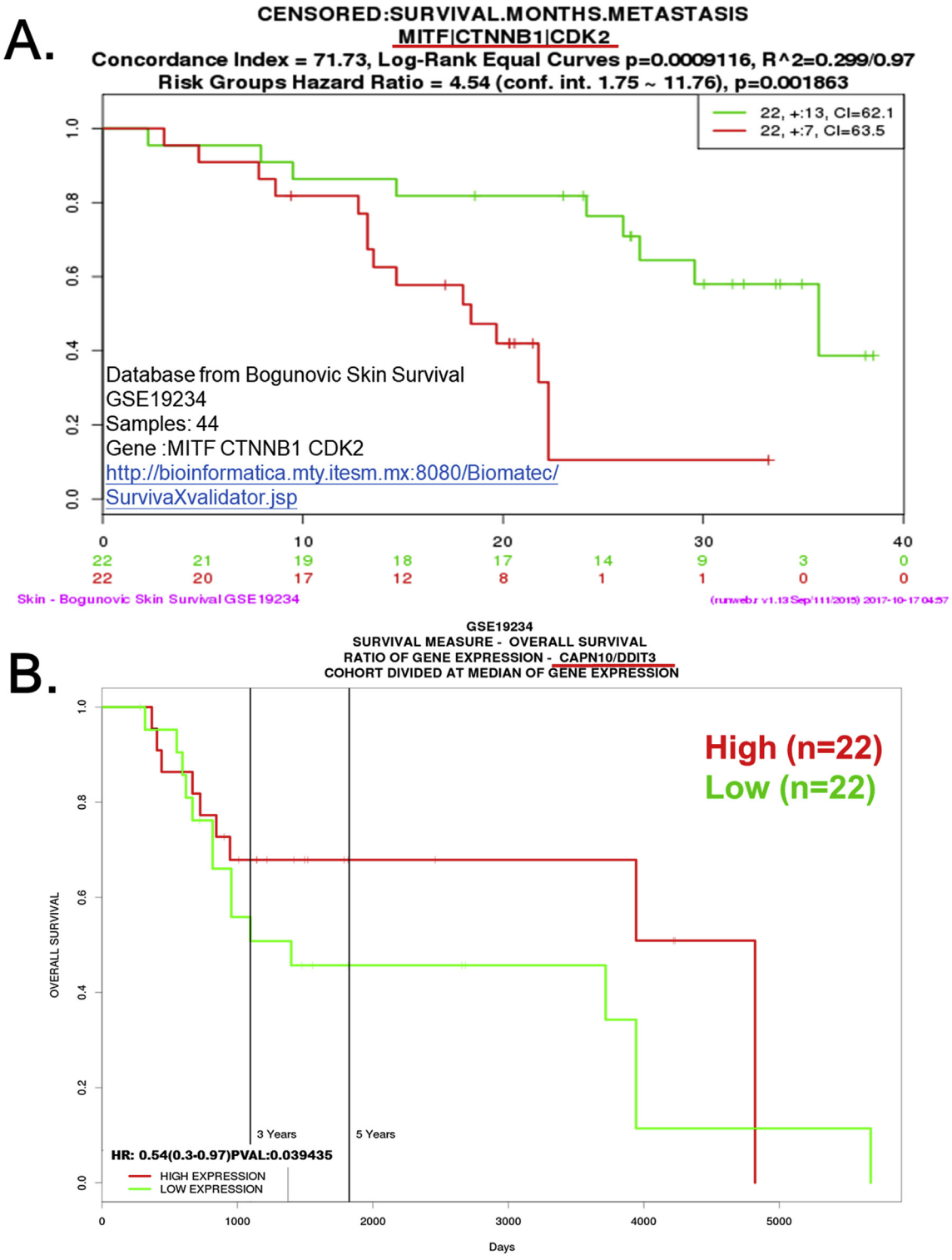

Fig. 7. High MITF, β-catenin, and CDK2 expressions were associated with decreased overall survival probability in melanoma. Data obtained were from the (A) Bogunovic Skin Survival dataset (GSE19234) through a comprehensive search using SurvExpress for MITF, CTNNB1 (β-catenin), and CDK2 evaluation, and (B) enhancements of the existing database PROGgeneV2 for CAPN10 (Calpain-10), DDIT3 (CHOP/GADD153).

induce growth inhibition and/or apoptosis after ER stress or DNA damage. As a transcription factor, it heterodimerizes with members of the C/EBP family of transcription factors. Moreover, it contains 2 prolines substituting for 2 residues in the basic region, critical for binding to DNA. All currently described functions of CHOP require the intact C-terminal DNA-binding and hetero-dimerization domain. Horndasch et al. have found that CHOP can function as a specific inhibitor of the Wnt/TCF pathway in Xenopus embryos as well as in mammalian embryonic and tumor cells. Their data demonstrates that the conserved N terminus of CHOP interacts with the N-terminal region of TCF, damaging the association of TCF to its DNA recognition site and restraining TCF-dependent transcriptional activity. Surprisingly, the inhibition of the Wnt-pathway depends on the very N-terminal domain of CHOP, whereas the leucine-zipper heterodimerization domain of CHOP is

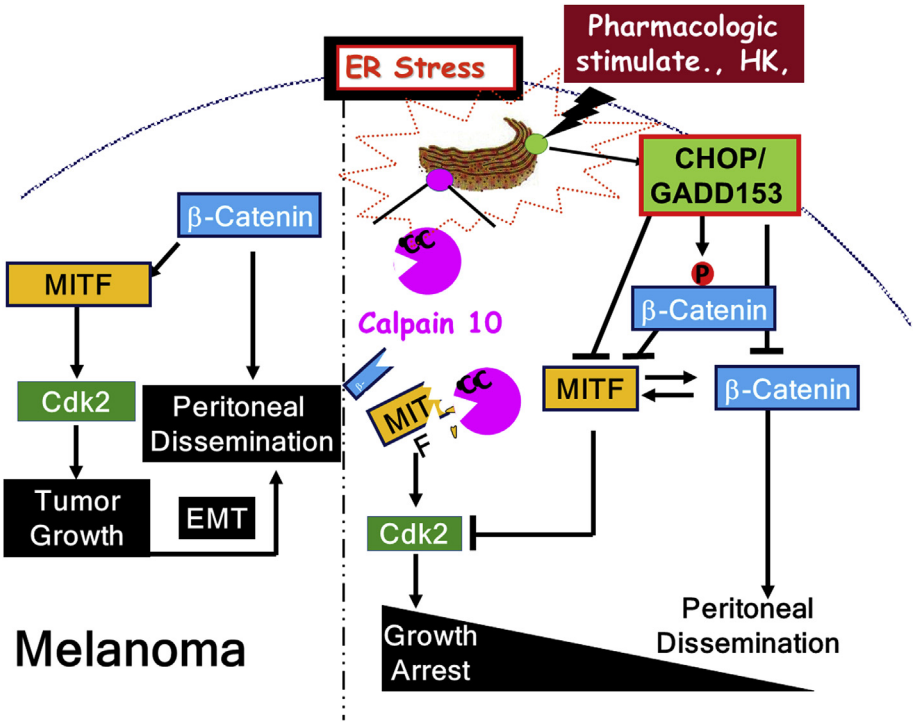

Fig. 8. Schematic of proposed mechanism for the role of Honokiol and blocked EMT and metastatic dissemination in melanoma. The schematic diagram represented Honokiol-induced ER stress and Calpain-10 activity, with subsequent MITF cleavage. Stimulation of CHOP/GADD153 activation with evoked β-catenin phosphorylation resulted in β-catenin degradation, triggering growth arrest and metastatic dissemination.

dispensable. These findings reveal a novel function of CHOP in its previously unknown role as a Wnt repressor, mediated by a novel molecular mechanism of CHOP. Similarly, the present study demonstrates that CHOP directly interacts with MITF and β-catenin and suppresses TCF-mediated transcription, while activating β-catenin phosphorylation. The findings suggest that Honokiol dictates CHOP to block Wnt activity and possibly, growth arrest, apoptosis, and EMT inactivation.

In conclusion, the current study demonstrates that Honokiol is a potent inhibitor of metastatic melanoma, especially the EMT program and cancer cell dissemination. Honokiol-induced CHOP is an inhibitor of the canonical Wnt pathway in metastatic melanoma cells and delineates a novel mechanism for this growth arrest gene. Honokiol-evoked CHOP also interacts with MITF and β-catenin, and inhibits the association of the TCF protein to DNA, further repressing Wnt-responsive genes. Future studies are warranted to further clarify the physiologic function of CHOP in quiescent stem cells and its role in patho-physiologic states.

Conflict of interest disclosure

The authors declare no conflicts of interest.

Conflicts of interest

None.

Conflicts of interest statement

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.026.

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