Two In-and-out Modulation Strategies for Endoplasmic Reticulum Stress-linked Gene Expression of Pro-apoptotic Macrophage-inhibitory Cytokine 1*

Seong-Hwan Park‡1, Hye Jin Choi‡1, Hyun Yang‡1, Kee Hun Do‡1, Juil Kim‡2, Hyun-Hong Kim‡2, Heejeong Lee‡1, Chang Gyu Oh‡, Dong Won Lee§, and Yuseok Moon‡¶2

From the ‡Laboratory of Systems Mucosal Biomodulation, Department of Microbiology and Immunology, and the ¶Department of Internal Medicine, Pusan National University School of Medicine, Yangsan 626-870, Korea and the §Research Institute for Basic Sciences and Medical Research Institute, Pusan National University, Busan 609-735, Korea

Background: Modulation of a pro-apoptotic MIC-1 was addressed under ER stress-linked alterations of intracellular components.

Results: Transporting of transcript-associated complex across cellular compartments is critical for MIC-1 mRNA stabilization.

Conclusion: Stabilization is due to complex formation with pumped-out RNA-binding protein and their timely exit from the stress granule.

Significance: Harmonized in-and-out modulation implicates key regulatory processes of MIC-1 expression in ER stress-linked therapy and biology.

Excessive and persistent insults during endoplasmic reticulum (ER) stress lead to apoptotic cell death that is implicated in a range of chronic inflammatory diseases and cancers. Macrophage inhibitory cytokine 1 (MIC-1), a member of the transforming growth factor-β superfamily, is diversely linked to the pathogenesis of cancer. To investigate the precise molecular mechanisms of MIC-1 gene regulation, ER stress and its related signals were studied in human colon cancer cells. Functionally, MIC-1 played pivotal roles in ER stress-linked apoptotic death, which was also influenced by C/EBP homologous protein, a well known apoptotic mediator of ER stress. ER stress enhanced MIC-1 mRNA stability instead of transcriptional activation, and there were two mechanistic translocations critical for mRNA stabilization. First, C/EBP homologous protein triggered protein kinase C-linked cytosolic translocation of the HuR/ELAVL1 (Elav-like RNA-binding protein 1) RNA-binding protein, which bound to and stabilized MIC-1 transcript. As the second critical in-and-out regulation, ER stress-activated ERK1/2 signals contributed to enhanced stabilization of MIC-1 transcript by controlling the extended holding of the nucleated mRNA in the stress granules fusing with the mRNA-decaying processing body. We propose that these two sequential in-and-out modulations can account for stabilized transcription and subsequent translation of pro-apoptotic MIC-1 gene in human cancer cells under ER stress.

The endoplasmic reticulum (ER) receives the global protein load during protein biosynthesis, conformational maturation, and modification. Various pathologic events, including abnormal protein production, viral infection, nutrient deprivation, xenobiotic-induced toxic stresses, or calcium depletion from the ER lumen, cause a particular type of stress called ER stress. An early event in the ER stress is the accumulation of misfolded/unfolded proteins that elicits unfolded protein responses in the cells (1, 2). In response to unfolded protein responses, phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) as a convergent point of the cellular stress response provides stress resistance by global protein translational arrest and induction of numerous stress-triggered cytoprotective genes (3, 4). Four different kinases are known to phosphorylate eIF2α, and particularly, ER-proximal sensors, such as RNA-dependent protein kinase (PKR)-related ER kinase (PERK), repress the global translation by phosphorylation of eIF2α, which limits the cellular supply for protein translation and provides cells with sufficient time to fix misfolded proteins from the ER stress. Along with PERK, cells also have additional mammalian eIF2α kinases, including PKR, general control non-derespressible-2 (GCN2), and heme-regulated inhibitor (HRI), depending on the types of cellular stress (4).

Excessive and persistent deleterious stress during ER stress leads to apoptotic cell death that is associated with a range of inflammatory, carcinogenesis, and metabolic diseases. Although the precise molecular mechanisms of ER stress-associated apoptosis are unknown, one of the representative involved factors is C/EBP homologous protein (CHOP), whose

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† These authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Microbiology and Immunology, Pusan National University School of medicine, Yangsan 626-813, Korea. Tel.: 82-51-510-8094; Fax: 82-55-382-8090; E-mail: moon@pnu.edu.

The abbreviations used are: ER, endoplasmic reticulum; eIF2α, eukaryotic translation initiation factor 2α; PKR, RNA-dependent protein kinase; PERK, PKR-related ER kinase; CHOP, C/EBP homologous protein; dnCHOP, dominant negative CHOP; nonsteriodal anti-inflammatory drug; TG, thapsigargin; ARE, AU-rich element; shHuR, HuR shRNA; P-body, processing body.
deficiency in vitro and in vivo causes the resistance to ER stress-induced cell death (5). CHOP induces downstream signals; growth arrest; and DNA damage-inducible gene 34, ER oxidase 1, death receptor 5, and carbonic anhydrase VI, which seem to be responsible for apoptosis. Recently, another possible mediator of cellular apoptosis in responses to ER stress, CHOP-regulated macrophage-inhibitory cytokine 1 (MIC-1) was implicated in drug-induced cancer cell death (6). MIC-1, also known as PTGF-β, PLAB, GDF15, PDF, NAG-1, and PLT4, is a transforming growth factor-β superfamily cytokine that is involved in epithelial tumor pathogenesis (7–9). Under normal resting conditions in epithelial cells, there is little or no detectable expression of MIC-1. However, with epithelial neoplastic transformation, MIC-1 expression rises dramatically and is further increased in response to a variety of anti-tumorigenic stimuli, such as γ irradiation, anti-inflammatory phytochemicals, and nonsteroidal anti-inflammatory drugs (NSAIDs) (10, 11). During the early stages of tumorigenesis, elevated MIC-1 can lead to tumor cell apoptosis, inhibition of blood vessel formation, and tumor cell cycle arrest (12). MIC-1 can be induced in either a p53-dependent or -independent way, and its inducible cellular signals also include other diverse growth-regulatory triggers (13–15).

In response to ER stress, eukaryotic cells selectively shut down the global protein translation via eIF2α phosphorylation, which results in a limited availability of the eIF2-GTP-tRNA\textsuperscript{Met} complex (16). When the translation initiation complex is stalled without eIF2, the 48 S complexes aggregate in a particular cellular structure called the stress granule. Stress granules assemble rapidly in response to stress and disappear slowly after its removal. The temporal storage in stress granules can provide mRNA with shelter from degradation and maintain silenced mRNAs to resume protein translation upon stress release. Export of mRNA from the nucleus, translation initiation, and mRNA stability are important control points in the post-transcriptional regulation of gene expression. Particularly, control of these processes is exerted through recognition of cis elements in the mRNA by specific binding proteins. Among mRNA-binding proteins, Elav-like RNA-binding proteins have been reported as a positive regulator of the AU-rich element (ARE)-containing mRNA stability. Four highly conserved Elav-like proteins have been identified: HuD, HuC, Hel-N1, and HuR. HuD, HuC, and Hel-N1 are expressed in terminally differentiated neurons and neuroendocrine tumors. In contrast, HuR, which has been studied most extensively, is ubiquitously expressed and predominantly localizes in the nuclear region. HuR can also shuttle between the cytoplasm and the nucleus, which mediates the export of specific types of ARE-containing mRNA (17, 18). HuR is also recruited into stress granules in association with translationally arrested mRNA, which is assumed to constitute protective shelter during stressful conditions against mRNA degradation.

In the present study, we evaluated the potential modulation of MIC-1 in human colon cancer cells under ER stress. Particular interest focused on the fate of MIC-1 mRNA at different regulatory steps with different translocation of signaling molecules across the cellular compartments in response to the ER stress. Critical modes of altered expression of the pro-apoptotic MIC-1 gene may provide new insights into intervention of human epithelial tumorigenesis.

**MATERIALS AND METHODS**

**Cell Culture Conditions and Reagents**—Intestinal cancer cell lines, HCT-116, HT-29, and HCT-8, were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 50 units/ml penicillin (Sigma-Aldrich), and 50 μg/ml streptomycin (Sigma-Aldrich) in a 5% CO\textsubscript{2} humidified incubator at 37 °C. The HCT-116 cell line has been extensively investigated as a representative model of human colon cancer in culture (19, 20). Cell number and viability were assessed by exclusion of trypan blue dye (Sigma-Aldrich) using a hemacytometer. Antibodies were purchased from the indicated companies as rabbit polyclonal anti-HuR, mouse monoclonal anti-phospho-ERK1/2, mouse monoclonal anti-CHOP (GADD153), goat polyclonal anti-MIC-1 (PTGF-β), rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit polyclonal anti-phospho-eIF2α (Assay Designs, Ann Arbor, MI), polyclonal anti-ERK1/2 antibody, and rabbit polyclonal anti-phospho-eIF2α (Cell Signaling Technology, Beverly, MA). Expression plasmid for short hairpin RNA (shRNA) of NAG-1 (pShNAG-1), dominant negative CHOP (dnCHOP), dominant negative PERK (dnPERK), and dominant negative PKR (dnPKR) were kindly provided from Dr. Thomas Eling (NIEHS, National Institutes of Health), Dr. Tomomi Gotoh (Kumamoto University) (21), Dr. J. Alan Diehl (Abramson Family Cancer Research Institute), and Pei-Jer Chen (National Taiwan University), respectively. Luciferase reporter containing MIC-1 promoter (−1739/+70) was kindly provided by Dr. Eling.

**Assessment of Apoptosis by Fluorescence-activated Cell Sorting (FACS)**—The DNA content of cells was determined by FACS analysis. Cells were plated in triplicate two or more times at 7.5 × 10\textsuperscript{5} cells/well in 60-mm diameter plates, incubated overnight, and then treated with serum-free medium for 24 h. After treatment, the cells were rinsed with phosphate-buffered saline (PBS), harvested, mixed with Annexin V-FITC in the dark for 15 min, centrifuged, and incubated with propidium iodine staining were classified as a group in a late apoptosis state.

**Western Immunoblot Analysis**—Levels of protein expression were compared using Western immunoblot analysis. Cells were washed with ice-cold PBS, lysed in boiling lysis buffer (1% (w/v) SDS, 1.0 mM sodium orthovanadate, and 10 mM Tris, pH 7.4), and sonicated for 5 s. Protein in the lysate was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Fifty micrograms of protein were separated by PVDF membrane (Amersham Biosciences). The blot was blocked for 1 h with 5% skim milk in Tris-buffered saline plus...
Tween 0.05% (TBST) and probed with the particular primary antibody for 2 h at room temperature or overnight at 4 °C. After washing three times with TBST, the blot was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and then washed three times with TBST. Protein was detected using enhanced chemiluminescence (ECL) substrate (Elipsis Biotech, Daejeon, Korea).

Traditional and Real-time RT-PCR—RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA (100 ng) from each sample was transcribed to cDNA by BD Sprint PowerScript (Clontech, Mountain View, CA). The amplification was performed with Hot Start ExTaq DNA polymerase (Takara Bio, Shiga, Japan) in a Mycycler thermal cycler (Bio-Rad) using the following parameters: denaturation at 94 °C for 2 min and 25 cycles of denaturation at 98 °C for 10 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.2% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. The 5′- and 3′-reverse complement PCR primers for amplification of each gene were as follows: human MIC-1, 5′-AGA TTC TGC CAG CAG TTG GT-3′ and 5′-AGA TTC TGCA GAA ACC AGG TT-3′. The relative amount of each mRNA was quantified by dividing by the density of the housekeeping gene. Each treatment includes three replicates to ensure statistical significance, and each independent experimental set was repeated two or three times. The relative quantification of gene expression was performed using the comparative Ct method. The Ct value is defined as the point where a statistically significant increase in the fluorescence has occurred. The number of PCR cycles (Ct) required for the FAM intensities to exceed a threshold just above background was calculated for the test and reference reactions. In all experiments, GAPDH was used as the endogenous control.

Transient and Stable Transfection—Cells were transfected with a mixture of plasmids using Trans-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer’s protocol. For transfection of the luciferase reporter gene, a mixture of 1.5 μg of firefly luciferase reporter and 0.15 μg of Renilla luciferase, pRL-null vector (Promega, Madison, WI) per 4.5 μl of Trans-LT1 reagent was applied to wells of a 6-well culture plate. For the luciferase assay, at 18 h after transfection, cells were exposed to chemicals for the next 24 h and lysed for the Dual-Luciferase reporter assay system (Promega). Transfection efficiency was maintained at around 50–60%, which was confirmed with pMX-enhanced green fluorescent protein (GFP) vector. To create pSilence and pSiEG1-expressing stable cell lines, cells were transfected using Trans-LT1 reagent. After 48 h, the cells were subjected to selection for stable integrants by exposure to 500 μg/ml G418 (Invitrogen) in complete medium containing 10% FBS. Selection was continued until monolayer colonies formed. The transfectants were then maintained in medium supplemented with 10% FBS and 250 μg/ml G418.

Luciferase Assay—Cells were washed with cold PBS, lysed with passive lysis buffer (Promega), and then centrifuged at 12,000 × g for 4 min. The supernatant was collected, isolated, and stored at −80 °C until assessment of luciferase activity. Luciferase activity was measured with a dual mode luminometer (model TD-20/20, Turner Designs, Sunnyvale, CA) after briefly mixing the supernatant (10 μl) with 50 μl of firefly luciferase assay substrate solution, followed with 50 μl of stop solution (Promega). Luciferase activity was normalized by dividing firefly luciferase activity by Renilla luciferase activity.

Construction of Plasmid—A CMV-driven small interference RNA (siRNA) was constructed by inserting the small hairpin RNA (shRNA) template into pSilencer 4.1-CMV-neo vector (Ambion, Austin, TX). The empty vector and shRNA of HuR, CHOP, PKR, and PERK insert-containing vector were designated pSilencer and pShHuR, respectively. Insert HuR, CHOP, PKR, and PERK shRNA targeted the sequence 5′-GTG CAA AGG GTT TGG CTT T-3′, 5′-AAG AAC CAG CAG AGG UCA CAA-3′, 5′-AAG CGA GAA ACU AGA CAA AGU-3′, and 5′-AAG TGA CGA AAT GGA ACA AGA-3′, respectively. The MIC-1 shRNA expression vector and luciferase reporter plasmid containing MIC-1 promoter (−1739/+70) were kindly provided by Dr. Jong-Sik Kim (Andong National University) and Dr. Eling Thomas (NIEMS, National Institutes of Health). Human MIC-1 cDNA was cloned into pcDNA 3.1-neo plasmid. dnCHOP and wild type CHOP constructs were provided by Dr. Tomomi Gotoh (Kumamoto University) (34), and FLAG-tagged wild type pexisome proliferator-activated receptor γ (PPARγ) plasmid was provided by Dr. Krishna Chatterjee (University of Cambridge). Expression plasmids for dominant negative PERK and dominant negative PKR were kindly provided by Dr. J. Alan Diehl (Abramson Family Cancer Research Institute) and Pei-Jer Chen (National Taiwan University), respectively. PERK cDNA expression plasmid was provided by Addgene (Cambridge, MA) (catalog no. 21814).

Confocal Microscopy—Cells were incubated in a glass bottom culture dish. After treatment with doxycylenovale or vehicle (dimethyl sulfoxide; DMSO), cells were fixed with 3.7% paraformaldehyde diluted in PBS. Fixed cells were permeabilized with 0.2% dimethyl sulfoxide; DMSO, cells were fixed with 3.7% paraformaldehyde diluted in PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. After 2 h of blocking with 3% bovine serum albumin (BSA) in PBS, cells were incubated with a 1:200 dilution in the same buffer of mouse polyclonal anti-HuR antibody (Santa Cruz Biotechnology, Inc.) at room temperature for 1.5 h and repeatedly washed using PBS. Incubation of Alexa Fluor 546 goat anti-mouse IgG (H+L) was done for 1.5 h at room temperature followed by repeated washes using PBS. After subsequent staining with 100 ng/ml 4',6-diamidino-2-phenylindole (DAPI) in PBS for 30 min, which has an absorbance at 405 nm, confocal images were obtained using a model FV1000 confocal microscope (Olympus, Tokyo, Japan) using single line excitation (546 nm) or multitrack sequential excitation (546 and 633 nm). Images were acquired and processed with FV10-ASW software.

RNA Fluorescence in Situ Hybridization—RNA fluorescence in situ hybridization was performed with modification of published protocol (22). Cells grown on chamber slides were fixed with cold 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in CSK (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES, pH 6.8) buffer. Slides were stored at 4 °C in 70% ethanol. Slides were dehydrated through an ethanol series to 100% ethanol, air-dried, and hybridized overnight at 37 °C. The MIC-1 mRNA (NCBI reference sequence NM_004864.2) probe was designed with the software program Primer3. The
primer sequence was 5′-ACT GCT GGC AGA ATC TTC GT-3′. Avidin-labeled probe in hybridization buffer (2× SSC, 0.5 mM EGTA, 50% dextran sulfate, 0.1% BSA, 20 mM vanadyl ribonucleoside) was denatured simultaneously by placing the slides on a 80 °C metal plate for 10 minutes. Hybridization with the probe was done at 37 °C overnight in a moist chamber. After hybridization, slides were washed three times in 50% formamide, 2× SSC, pH 7.0, for 5 min each at 42 °C, three times in 2× SSC for 3 min, and once in PBS at room temperature. Avidin-labeled probe was detected with streptavidin Alexa Flour 405 conjugate (1:250) (Invitrogen). Antibodies were diluted in PBS containing 3% (w/v) BSA and were incubated for 30 min at 37 °C. After antibody incubation, the slides were washed three times with 4× SSC containing 0.1% Tween 20. After staining with 100 ng/ml DAPI in PBS for 30 min, confocal images were obtained using an Olympus FV1000 confocal microscope (Olympus). Images were acquired and processed with FV-10-ASW software.

**Cell Survival Analysis**—One thousand cells were plated on a 60-mm diameter dish for 15 days for focus formation assays. First, cells were exposed to thapsigargin for 48 h and incubated for 12 days at 37 °C in thapsigargin-free complete medium in a fully humidified atmosphere of 5% CO₂ in air. Cells were then washed with PBS, fixed with 100% methanol, stained, and visualized with Karyomax Giemsa solution (Invitrogen) for 2 min, followed by successive washes with PBS at room temperature to visualize colony growth. Cells were counted from 10 randomly selected 2-cm² grids/well.

**RNA-Chromatin Immunoprecipitation (RNA-ChIP)**—RNA-ChIP is a method to examine RNA-protein interactions. RNA-ChIP is performed in a similar way as a regular chromatin immunoprecipitation assay with an extra step of adding RNase inhibitor to protect RNA after cross-linking and the pulled down RNA is detected by RT-PCR. Immunoprecipitation of protein-RNA complexes was performed by a modified protocol for chromatin immunoprecipitation. Briefly, HCT-8 cells were seeded at 2.5 × 10⁶/100-mm diameter dish with complete RPMI 1640 medium and grown for 24 h. After treatment with deoxynivalenol or DMSO, protein and RNA were cross-linked with 1% formaldehyde. The cytoplasmic extract was incubated at 4 °C overnight with 5 µg of either rabbit anti-rabbit IgG (non-specific control) or an antibody against HuR. The antibody-bound complex was precipitated with protein A-Sepharose beads, which were sequentially washed once for 5 min with low salt buffer, high salt buffer, LiCl buffer, and TE buffers. The protein-RNA complex was eluted from the protein A-Sepharose beads with 250 µl of elution buffer at 37 °C for 15 min. The RNA in the immunoprecipitated complex was released by reversing the cross-linking at 65 °C for 4–5 h with 200 mM NaCl and 20 µg of proteinase K. RNA was then extracted from the solution using TRIzol reagent and subjected to RT-PCR.

**Statistical Analyses**—Data were analyzed using SigmaStat for Windows (Jandel Scientific, San Rafael, CA). For comparison of two groups of data, Student’s t test was performed. For comparison of multiple groups, data were subjected to analysis of variance, and pairwise comparisons were made by the Student-Newman-Keuls method. Data not meeting the normality assumptions were subjected to Kruskal-Wallace analysis of variance on ranks, and then pairwise comparisons were made by the Student-Newman-Keuls method.

**RESULTS**

**ER Stress-induced MIC-1 Is Linked with Cancer Cell Apoptosis**—In the present study, MIC-1 was assessed as a pro-apoptotic mediator in response to ER stress that was induced by representative chemical triggers, including thapsigargin (TG) and tunicamycin, in human intestinal cancer cells. The chemical-induced ER stress increased MIC-1 gene expression in HCT-116 human colonic adenocarcinoma cells (Fig. 1A). Moreover, MIC-1 induction by ER stress was observed in other epithelial cancer cells, such as HCT-8 and HT-29 cells (Fig. 1, B and C), and the chemical-induced ER stress increased MIC-1 gene expression in a dose-dependent manner (Fig. 1D). Another ER stress trigger, tunicamycin, also enhanced MIC-1 production in HCT-116 cells (Fig. 1F). Moreover, MIC-1 mRNA was also enhanced by chemical ER stressor in a time-dependent way (Fig. 1F). Along with the increase of intracellular MIC-1 expression, MIC-1 as a cytokine was also released from the cells exposed to chemical ER stress (Fig. 1G). Functionally, MIC-1 protein was tested for its involvement in ER stress-induced apoptosis in the intestinal tumor cells. TG treatment increased tumor cell death, and furthermore MIC-1 suppression using shRNA attenuated the apoptotic response to ER stress (Fig. 1H). As a well known mediator of ER stress-induced apoptosis, CHOP was shown to be also involved in the programmed death by ER stress. The flow cytometry data were in agreement with the results of the DNA fragmentation assay as an early indication of apoptosis (Fig. 1I). Moreover, ER stress increased chromosomal condensation with an increasing number of nuclear fragmentations (Fig. 1J), which were decreased by suppression of MIC-1 or CHOP protein. In a tumor cell colony-forming assay in the presence of chemical ER stress, MIC-1 suppression increased numbers of surviving colonies (Fig. 1K), suggesting that MIC-1 mediates tumor cell death in response to ER stress. Taken together, the observations indicate that MIC-1 plays pivotal roles in intestinal tumor cell death by ER stress.

**ER Stress Triggers MIC-1 Gene Expression via eIF2α Kinases, CHOP, and ERK1/2**—The chemical ER stressors enhanced the phosphorylation of eIF2α (Fig. 2A), which is a highly conserved point of convergence for diverse stressful conditions, including ER stress (3, 4). PERK represses the translation by phosphorylation of eIF2α, allowing cells to have sufficient times to fix the misfolded proteins from the ER stress. Chemical ER stress activated the phosphorylation of eIF2α, which was suppressed by dominant negative PERK expression or by PERK shRNA expression and increased by wild type PERK expression (Fig. 2B). More importantly, PERK was positively involved in ER stress-induced MIC-1 expression in the colon cancer cells. PKR is another eIF2α kinase whose repression using the dominant negative form or PKR shRNA also alleviated ER stress-activated MIC-1 protein and phosphorylation of eIF2α (Fig. 2C). As an apoptosis-associated target of the eIF2α kinase signaling cascade, CHOP protein was also assessed for its involvement in MIC-1 induction by ER stress. Suppression of CHOP using expression of dnCHOP or CHOP shRNA decreased MIC-1
FIGURE 1. Induction of MIC-1 protein by chemical ER stress in human intestinal cancer cells. A–C, cells (HCT-116, HCT-8, and HT-29) were treated with 0.5 μM TG for the indicated times. D, HCT-116 cells were treated with each dose of TG for 24 h. E, HCT-116 cells were treated with 5 μg/ml tunicamycin for the indicated times. Total cell lysate was subjected to Western blot analysis. F, measurement of MIC-1 mRNA using conventional RT-PCR in HCT-116 cells exposed to vehicle or 0.5 μM TG for each time. G, HCT-116 cells were treated with 0.5 μM TG for the indicated times. Incubated culture media were collected for extracellular protein analysis. H, vector-, short hairpin RNA MIC-1 expression plasmid (shMIC1)-, dominant negative CHOP expression plasmid (dnCHOP)-, or MIC-1 expression plasmid (MIC1)-transfected cells were treated with 0.5 μM TG for 24 h. Apoptosis was quantified using the Annexin V/FITC staining method. *, significant difference from each control group (vehicle- or TG-treated control group) (p < 0.05). I, vector-, shMIC1 or dnCHOP expression plasmid-transfected cells were treated with 0.5 μM TG for 24 h, and then the cellular fragmented DNA was analyzed. J, vector-, short hairpin RNA MIC-1 expression plasmid- or shMIC1 expression plasmid-transfected cells were treated with 0.5 μM TG, and then nuclei were visualized with DAPI staining. K, vector-, short hairpin RNA MIC-1 expression plasmid-, or MIC-1 expression plasmid-transfected cells were treated with TG, and then the colony forming assay was performed. *, significant difference from the control group (p < 0.05). Error bars, S.E.
Dynamics of ER Stress-modulated MIC-1

A

| TG | Tunicamycin (min) |
|----|-------------------|
| 0  | 10                |
| 10 | 30                |
| 30 | 0                 |

p-eIF2α density relative to actin

actin

B

| Control | dnPERK | shPERK | wtPERK |
|---------|--------|--------|--------|
| +       | +      | +      | +      |
| +       | +      | +      | +      |
| +       | +      | +      | +      |

MIC-1 density relative to actin

Actin

p-eIF2α density relative to actin

Actin

C

| Control | dnPKR | shPKR | wtPKR |
|---------|-------|-------|-------|
| -       | +     | -     | +     |
| -       | +     | -     | +     |
| -       | +     | -     | +     |

MIC-1 density relative to actin

Actin

p-eIF2α density relative to actin

Actin

D

| Control | dnCHOP | shCHOP |
|---------|--------|--------|
| -       | +      | -      |
| -       | +      | -      |
| -       | +      | -      |

MIC-1 density relative to actin

Actin

CHOP density relative to actin

Actin

E

| Control | U0126 (µM) |
|---------|------------|
| +       | 0          |
| +       | 0          |
| +       | 1          |
| +       | 5          |
| +       | 10         |
| -       | 10         |

MIC-1 density relative to actin

Actin

p-ERK1/2 density relative to ERK1/2

ERK1/2

F

| control | dnPERK | control | dnPKR | control | shPERK | control | shPKR | TG |
|---------|--------|---------|-------|---------|--------|---------|-------|----|
| -       | +      | +       | -     | +       | -      | +       | +     | +  |
| -       | +      | +       | -     | +       | -      | +       | +     | +  |

G

| Control | dnCHOP |
|---------|--------|
| +       | +      |
| +       | +      |

MIC-1 density relative to actin

CHOP density relative to actin
expression (Fig. 2D), indicating positive regulation of MIC-1 induction by CHOP. After assessing the inhibitory effect of various signaling inhibitors on MIC-1 induction by ER stress (data not shown), only ERK1/2 inhibition using MEK1/2 inhibitor U0126 was proven to attenuate MIC-1 induction as well as ERK1/2 phosphorylation, implicating the involvement of ERK1/2 signals in gene expression (Fig. 2E). In particular, chemical ER stressor enhanced ERK1/2 phosphorylation, and ER stress-linked eIF2α kinases, including PERK and PKR, were involved in activation of the ERK1/2 signaling pathway (Fig. 2F). Moreover, dual suppression of CHOP and ERK1/2 action led to almost complete suppression of MIC-1 induction by tunicamycin, another chemical ER stressor (Fig. 2G). Taken together in terms of signa-ling pathway, ER stress-related mediators and ERK1/2 signals were positively involved in MIC-1 induction in human epithelial cancer cells.

Enhanced MIC-1 Expression Is Associated with MIC-1 mRNA Stabilization by ER Stress—The effect of ER stress on MIC-1 gene expression was assessed at levels of transcriptional or post-transcriptional regulation. Cells transfected with MIC-1 promoter-linked reporter plasmid showed only a marginally increased level of transcriptional activity in the presence of ER stress over the control, whereas the steady level of MIC-1 mRNA was 3 times enhanced by ER stress (Fig. 3A). In contrast to the transcriptional level, chemical ER stress enhanced MIC-1 mRNA stability. The chemical ER stressor TG augmented the half-life of MIC-1 mRNA, which was significantly diminished in cells expressing the dominant negative form of CHOP or ERK1/2-inhibited cells (Fig. 3, B–D). Therefore, it can be suggested that ER stress-linked CHOP and ERK1/2 signals were positively involved in stabilization of MIC-1 mRNA. Stabilization of MIC-1 transcript by chemical-induced ER stress was confirmed using a CMV promoter-activated luciferase reporter.
gene tagged with the 3′-untranslated region (3′-UTR) of MIC-1 transcript (Fig. 4A). The chemical ER stress increased reporter gene expression in a dose-dependent manner. MIC-1 3′-UTR contains four AREs that are involved in mRNA stability. By contrast, ER stress-mediated induction of another reporter gene devoid of MIC-1 AREs was strongly attenuated when compared with luciferase activities of the reporter with a full-length MIC-1 3′-UTR. A number of RNA stability-regulating proteins can bind to this ARE. Among many RNA-binding proteins regulating mRNA stability, the HuR protein is considered as a definite enhancer of mRNA stability (23). Moreover, The mRNA-stabilizing action of HuR has also been addressed using chimeric mRNAs with the ARE (24). When HuR expression was deleted using shRNA, the cellular MIC-1 level was almost...
completely suppressed (Fig. 4B), indicating a positive regulation of MIC-1 expression by HuR. Moreover, ER stressor-augmented stability of MIC-1 mRNA was significantly diminished in cells expressing the HuR shRNA (shHuR) (Fig. 4C). Therefore, it can be suggested that ER stress-linked HuR proteins were positively involved in stabilization of MIC-1 mRNA. Particularly, ER stress enhanced the level of MIC-1 mRNA bound to HuR, which was attenuated in cells with the dominant negative form of CHOP (Fig. 4D). Moreover, ERK1/2 was also positively involved in binding of HuR to MIC-1 mRNA during ER stress response in the cancer cells (Fig. 4E). Because MIC-1 was linked to the apoptotic cell death by ER stress, MIC-1-stabilizing HuR was tested for its involvement in stress-mediated cell death. When HuR level was suppressed using its shRNA expression, TG-induced apoptotic DNA fragmentation was decreased (Fig. 4F). HuR-linked cell death was also confirmed by the observation of chromosomal condensation and fragmentation as another apoptosis index (Fig. 4G). Additionally, the flow cytometry data were in agreement with the results of the DNA fragmentation assay as an indication of apoptosis (Fig. 4H). Taken together, HuR protein mediated stabilization of pro-apoptotic MIC-1 transcript during ER stress in cancer cells.

**CHOP Modulates In-and-out Behavior of HuR Protein by Limiting Peroxisome Proliferator-activated Receptor γ Expression**—Because CHOP is a well known dominant negative factor of C/EBPβ (25), C/EBPβ downstream targets, including PPARγ, can be suppressed (26, 27). This negative relationship during the ER stress was proven in our recent report that ER stress-induced CHOP suppresses PPARγ expression (28). In the present study, suppression of CHOP using CHOP shRNA expression enhanced PPARγ expression in response to chemical ER stress TG, indicating regulatory action of CHOP on PPARγ levels (Fig. 5A). Although PPARγ is generally involved in transcriptional regulation, it is also a critical player in post-transcriptional modulation by attenuating cytosolic translocation of HuR protein (29, 30). When PPARγ expression was exogenously enhanced, ER stress-triggered cytosolic translocation of HuR protein was strongly attenuated (Fig. 5B). Therefore, ER stress-induced CHOP protein would allow more cytosolic translocation of HuR protein by limiting PPARγ expression, which contributed to stabilization of MIC-1 transcript, as shown in Figs. 3 and 4. Furthermore, the mechanism of PPARγ-mediated regulation of HuR translocation was investigated in detail. As one potent target of PPARγ action (31), protein kinase Cα (PKCα) was addressed for its involvement in HuR translocation. First, inhibition of PKCα strongly suppressed ER stress-induced cytosolic translocation of HuR (Fig. 5C), suggesting the possibility that phosphorylation signals can affect the localization of HuR protein under ER stress. ER stress activated PKCα, which then translocated into the nuclear region, but exogenously introduced PPARγ-bound to phosphorylated PKCα, which was retained in the cytoplasm (Fig. 5D). Therefore, PPARγ suppressed nuclear translocation of the active form of PKCα, which blocked phosphorylation and translocation of nuclear HuR in response to ER stress. These can account for the finding that PPARγ expression attenuated ER stress-triggered cytosolic translocation of HuR protein as shown in Fig. 5B as well. It was thus tested whether PPARγ and PKCα as HuR regulators were involved in the MIC-1 induction and mRNA stabilization by the ER stressor. PPARγ expression and PKC inhibition using GF 109203X (GF) decreased ER stress-induced MIC-1 expression (Fig. 5E) and stabilization of MIC-1 transcript (Fig. 5F). Taken together, PPARγ expression attenuated cytosolic translocation of HuR protein by blocking PKC, which contributed to decreased stability of MIC-1 mRNA and subsequent reduction of MIC-1 protein expression.

**Timely In-and-out Modulation of Stress Granule Is Critical for MIC-1 mRNA Stability**—HuR moved out of the nuclei in response to the chemical ER stress, but the cytosolic translocation was attenuated by suppression of CHOP (Fig. 6A). This event can be easily expected because CHOP is a positive modulator of the cytosolic translocation of HuR via PPARγ and PKCα. In contrast, signaling inhibition of the ERK1/2 pathway did not decrease the cytosolic translocation of HuR, although the ERK1/2 signal is importantly involved in HuR-mediated stabilization of MIC-1 mRNA in response to ER stress. Therefore, only the simple cytosolic translocation of HuR and subsequent stabilizing binding to mRNA cannot explain the ERK1/2-mediated stabilization of MIC-1 transcript. Cellular localization of HuR protein during early ER stress was sequestered to stress granules, which is a critical step for the protection of mRNA against the stressful environment. Protected nucleated mRNA can then escape from the stress granules and reinitiate translational synthesis after the stress release. ER stress transiently increased formation of HuR-containing stress granules, but CHOP-suppressed cells did not form the stress granules, and HuR was limited to the nuclear region (Fig. 6B). In contrast, ERK1/2 inhibition extended the presence of HuR-containing stress granules (Fig. 6, B and C), suggesting ERK1/2-mediated negative regulation of the extended retention of HuR-entangled mRNA in the stress granules. The prolonged formation of the stress granules contained TIA-1 co-localized with HuR protein. However, whereas extended retention of gene transcript in the stress granules

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**FIGURE 4. Stabilization of MIC-1 mRNA by bound HuR.** A, cells were transfected with vector of the CMV-promoted luciferase gene tagged with full-length (241 base pairs) MIC-1-3′-UTR or tagged with ARE-deleted MIC-1-3′-UTR (113 base pairs). Then cells were treated with TG for 9 h for luciferase assay, * significant difference from the group of full-length MIC-1-3′-UTR at each dose of TG (p < 0.05). RLU, relative luciferase units. B, vector- or shHuR expression plasmid-transfected cells were treated with 0.5 μM TG for 24 h. Total cell lysate was subjected to Western blot analysis. C, vector- or shHuR expression plasmid-transfected cells were treated with 0.5 μM TG for 9 h to reach the maximum level of MIC-1 mRNA, and then its transcription was terminated by adding 5 μM actinomycin D. Total RNA was analyzed using the RT-PCR method. The different letter above each bar represents significant difference between two groups (p < 0.05). D and E, vector- and dnCHOP expression plasmid-transfected cells were pretreated with vehicle or 5 μM U0126 and then treated with 0.5 μM TG for 45 min, and RNA-bound HuR protein was immunoprecipitated. The extracted RNA was measured using RT-PCR to measure MIC-1 transcripts bound to HuR protein. The different letter above each bar represents significant difference between two groups (p < 0.05). F, vector- or short hairpin RNA HuR (shHuR) expression plasmid-transfected cells were treated with 0.5 μM TG for 24 h, and then the cellular fragmented DNA was analyzed. G, vector- or shHuR expression plasmid-transfected cells were treated with 0.5 μM TG for 24 h, and then nuclei were visualized with DAPI staining. H, vector- or shHuR expression plasmid-transfected cells were treated with 0.5 μM TG or 5 μM/ml tunicamycin for 24 h. Apoptosis was quantified using the FITC staining method, * significant difference from each control group (p < 0.05). Error bars, S.E.
delays reinitiation of translation, the contents of the prolonged stress granules are easily remodeled and are exposed to the processing bodies (P-bodies). In the present study, ERK1/2-inhibited cells showed extended formation of stress granules, and it is thus necessary to observe the association with P-bodies in the presence of ER stress. In Fig. 6D, cells under ER stress showed the formation of the stress granules containing MIC-1 transcript, but the stress granules were also accompanied by the P-bodies. However, after the stress release (3.0 h after chemical insult), the stress granules disappeared. By contrast, cells exposed to ERK1/2 inhibitor and ER stress had extended formation of the stress granule-MIC-1 transcript-P-body complex. Therefore, ERK1/2 inhibition delayed the exit of nucleated MIC-1 transcript from the stress granules and enhanced the exposure of MIC-1 transcript to mRNA-decaying P-bodies, which can account for the reduced stabilization of MIC-1.

FIGURE 5. PPARγ-mediated retention of nuclear HuR protein via PKCα regulation. A, empty vector- or dnCHOP-expressing cells were treated with 0.5 μM TG for 4 h, and each mRNA was measured using RT-PCR. Images in the box at the bottom are representative data for the quantification. The different letter above each bar represents significant difference between two groups (p < 0.05). B, empty vector- or WT PPARγ-expressing cells were treated with 0.5 μM TG for 1.5 h. Cytoplasmic and nuclear proteins were analyzed using Western blot analysis. C, cells were pretreated with DMSO or PKC inhibitor GF 109203X (GF) and then treated with 0.5 μM TG for 1.5 h. Cytoplasmic and nuclear proteins were analyzed using Western blot analysis. D, empty vector- or WT PPARγ-expressing cells were treated with 0.5 μM TG for 1.5 h. Cytoplasmic and nuclear proteins were analyzed using immunoprecipitation (IP) and Western blot analysis. p-PKC, phospho-PKC. E, empty vector- or WT PPARγ-expressing cells were pretreated with DMSO or PKC inhibitor GF 109203X and then treated with 0.5 μM TG for 24 h. Total cell lysate was subjected to Western blot analysis. F, empty vector- or WT PPARγ-expressing cells were pretreated with DMSO or PKC inhibitor GF 109203X and then treated with 0.5 μM TG for 9 h to reach the maximum level of MIC-1 mRNA, and then its transcription was terminated by adding 5 μM actinomycin D. Total RNA was analyzed using the RT-PCR method. The different letter above each bar represents significant difference between two groups (p < 0.05). Error bars, S.E.
mRNA by ERK1/2 inhibition in the data of Figs. 5 and 6. Taken together, the present data indicate that the ERK1/2 signal is needed for regulation of extended formation of stress granules and subsequent fusion with the P-body. It can be thus concluded that the ERK1/2-associated process is favorable for the shift to the reinitiation of MIC-1 expression by avoiding P-body-mediated degradation in the stress granules (Fig. 7).

DISCUSSION

The present study demonstrates that tumor cells under ER stress produce pro-apoptotic MIC-1 protein due to mRNA regulation via two in-and-out translocation mechanisms. The stress-triggered gene induction is a pattern of cellular response along with global translational arrest of constitutively expressed genes. ER stress also shuts down protein synthesis via phosphorylation of eIF2α, leading to stalling of the translation initiation complex without eIF2 and formation of stress granules. HuR is recruited into the stress granules in association with translationally arrested mRNA, which constitutes a protective shelter during cellular stress against mRNA degradation. In the present study, ER stress-induced CHOP mediated cytosolic translocation of HuR and formation of stress granules. HuR positively affected MIC-1 gene regulation by stabilizing its mRNA during ER stress. MIC-1 expression is modulated at various levels, including both transcriptional and post-transcriptional regulation (6, 32, 33). Mechanistically, CHOP-mediated cytosolic translocation of HuR was a critical factor for MIC-1 mRNA stabilization. Shuttling of the HuR protein across the nuclear membrane is regulated by different signaling molecules, including different members of the mitogen-activated protein kinase (MAPK) family, the AMP-activated kinase family, cyclin-dependent kinases (CDK1 or -2), and protein kinase C (PKC). In particular, p38 MAPK and PKC/δ enhance the ATP-dependent export of HuR protein via phosphorylation (34–36). Our results also suggested PKC/δ and PPARγ as mechanistic links between CHOP and HuR regulation. ER stress-induced CHOP is basically a negative modulator of PPARγ.

FIGURE 6. Cellular dynamics of ERK1/2-linked MIC-1 gene expression in tumor cells under ER stress. A, vector- and dnCHOP expression plasmid-transfected cells were pretreated with vehicle or 5 μM U0126 and then treated with 0.5 μM TG for 1.5 h. Measured cytoplasmic HuR represents a relative value of density of HuR protein per housekeeping protein in the cellular lysate. B, vector- and dnCHOP expression plasmid-transfected cells were pretreated with vehicle or 5 μM U0126 and then treated with 0.5 μM TG for 1.5 h. Stress granules stained with HuR protein per cell were counted (B) or visualized and enumerated by confocal microscopy (C). The different letter above each bar represents a significant difference between two groups (p < 0.05). C, cells were pretreated with vehicle or 5 μM U0126 and then treated with 0.5 μM TG. Stress granules stained with HuR and TIA1 protein per cell were visualized by confocal microscopy. Images were observed at ×3,600 magnification. D, cells were pretreated with vehicle or 5 μM U0126 and then treated with 0.5 μM TG for 1.5 or 3 h. Staining of MIC-1 transcript (blue), stress granules (HuR protein; red), and P-bodies (DCP-1 protein; green) were visualized by confocal microscopy. Images were observed at 12,000× magnification. Error bars, S.E.

FIGURE 7. A putative mechanism of ER stress-induced MIC-1 expression in human intestinal cancer cells. ER stress induces phosphorylation of eIF2α (P-eIF2α), which results in translational arrest mediated by PERK and PKR. Downstream effectors, including CHOP and ERK1/2, are positively involved in MIC-1 induction at the post-transcriptional levels. Mechanistically, CHOP is related with cytosolic translocation of HuR protein stabilizing MIC-1 mRNA. By contrast, ERK1/2 signals also contribute to MIC-1 mRNA stabilization by regulating prolonged stress granule formation, its association with mRNA-decaying P-body, and reinitiation of translation. Successfully translated MIC-1 then can contribute to intestinal epithelial tumor cell apoptosis.
expression. Our previous investigation suggests that CHOP protein interferes with the homodimeric C/EBPβ, which is a critical transcriptional activator of PPARγ induction (28). Otherwise, enhanced PPARγ bound to and detained the active form of PKCα in the cytoplasm. Therefore, ER stress-triggered CHOP controls PPARγ and allows nuclear translocation of the active PKCα protein. PKC-mediated phosphorylation and cytosolic translocation of HuR protein contributed to the enhanced stabilization of MIC-1 mRNA in response to ER stress in the present study. It was also reported that PKC is involved in ribotoxin-triggered HuR translocation (6) and that PPARγ inhibits the PKC signaling cascade by limiting membrane translocation (31). Our recent published study (28) and the present result also support a negative regulation of HuR translocation by PPARγ, which is suppressed by CHOP expression (Fig. 7).

Although stress granules are a transient shelter from an RNA-degrading environment under stressful conditions, the protected transcripts are exported to the cytoplasm for reinitiation of translation or are translocated to an adjacent P-body for degradation. In the present study, inhibition of the ERK1/2 signaling pathway extended the presence of stress granules containing HuR protein. However, ERK1/2 inhibition suppressed stress-mediated stabilization of MIC-1 transcript and even decreased HuR binding to MIC-1 transcript. Therefore, it indicates that ERK1/2 signals can be involved in export of MIC-1 mRNA from the stress granules and prompt reinitiation of MIC-1 translation in cells that recover from ER stress. Extended formation of stress granules by ERK1/2 inhibition increased the contact of stress granules with P-bodies for mRNA degradation. MIC-1 transcript over-ohold in the stress granule can undergo degradation process by support of the neighboring P-body. In particular, recent reports suggested that MIC-1 mRNA stabilization is mediated by drug-activated signaling pathways, such as ERK1/2 signals (6, 32). Stabilization of ER stress-induced MIC-1 mRNA was also positively linked with ERK1/2 activation in the present study. Particularly, ERK1/2-linked mRNA stabilization during ER stress indicates avoidance of P-body-mediated degradation and reinitiation of translation. Therefore, an extended stay of the MIC-1 mRNA in a stress granule-associated HuR does not simply guarantee the high production of MIC-1 protein. Protected MIC-1 transcript needs to be released from the stress granule for reinitiation of translation after recovery from the stress without delay.

Functionally, MIC-1 expression was positively associated with epithelial tumor cell death in the present study. MIC-1 has been suggested to play a role in the anti-tumor activity of chemopreventive agents like NSAIDs and anti-inflammatory natural products. Moreover, our recent study reported that NSAID-triggered cellular stress is positively associated with MIC-1-mediated cell death (6). Mechanistically, MIC-1-enhancing CHOP has been extensively studied for its pro-apoptotic action in different studies. Although target genes of CHOP, such as carbonic anhydrase VI, a Ternn/Odz homologue, and actin-binding protein have been identified (37, 38), none is directly involved in death or survival responses. The present study implicates CHOP-regulated MIC-1 in the mediation of ER stress-induced apoptosis in colon cancer cells. A recent study demonstrated that MIC-1 protein enhances expression of death receptors, including DR4 and DR5, in epithelial tumor cells (39). Moreover, the DR pathway activates caspase-3-dependent DNA fragmentation via activation of poly(ADP)-ribose polymerase, which occurs in apoptotic cell death due to ER stress-induced CHOP and subsequent induction of DR5 gene expression (40). In addition to the intracellular action of CHOP-mediated MIC-1, secreted MIC-1 can influence cells via receptor-linked signaling pathways. Members of the TGF-β superfamily conventionally bind to transforming growth factor receptor, which transduces signals via Smad proteins. Acute treatment of MIC-1 protein enhances phosphorylation of Smad1/2/3/5/8 (41). Moreover, TGF-β-activated Smad triggers apoptotic cell death by enhancing death-associated protein kinase or growth arrest and DNA damage-inducible protein 34 (GADD34) (42, 43). Despite the anti-tumor activity of MIC-1, the protein can be associated with the ER stress-mediated cytotoxic effects in the normal gastrointestinal epithelial tissues. ER stress is involved in ulcerative diseases, including ulcerative colitis and drug-induced gastric ulceration (44–46). In particular, ER stress-activated CHOP enhances intestinal mucosal cell apoptosis by down-regulating Bcl-2 and activating caspase-11-linked signaling pathways. Normal colorectal mucosal epithelial cells induce apoptotic cell death by enhancing MIC-1 expression in mice orally exposed to the NSAID sulindac sulfide, which induces ER stress responses (6, 10). Therefore, pro-apoptotic MIC-1 plays key roles in ER stress-induced epithelial cytotoxicity. Conclusively, ER stress-enhanced MIC-1 protein can be closely associated with intestinal cancer cell death in the present study, but this requires further careful investigation because MIC-1 is also involved in the normal cellular cytotoxicity. Moreover, enhanced MIC-1 production in tumorigenesis is not always beneficial. MIC-1 has been linked to the modulation of cellular response of migrating cells in the extracellular matrix and circulation (47, 48). In colon cancer, increasing MIC-1 expression is also associated with the progression of colonic adenomas to invasive cancer and subsequent metastasis (7). In the case of epithelial cancer, serum MIC-1 levels increase with the progression of tumors to metastasis (7, 49, 50). Because MIC-1 particularly facilitates the migration of the transformed cells, chronically induced MIC-1 can facilitate dispersal of tumor cells in the circulation and subsequent metastasis, suggesting a potent involvement of ER stress responses in tumor progression in cancer patients. Taken together, up-regulated MIC-1 during ER stress can be either harmful or beneficial during overall tumorigenesis and inflammation. It is thus necessary to obtain more systematic observation and assessment of MIC-1 regulation in human intestinal epithelial pathogenesis.

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