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SS-A/Ro52 promotes apoptosis by regulating Bcl-2 production

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ABSTRACT

SS-A/Ro52 (Ro52), an autoantigen in systemic autoimmune diseases such as systemic lupus erythematosus and Sjögren's syndrome, has E3 ligase activity to ubiquitinate proteins that protect against viral infection. To investigate Ro52's role during stress, we transiently knocked it down in HeLa cells by siRo52 transfection. We found that Ro52^{low} HeLa cells were significantly more resistant to apoptosis than wild-type HeLa cells when stimulated by H_2O_2- or diamide-induced oxidative stress, IFN-α, IFN-γ and anti-Fas antibody, etoposide, or γ-irradiation. Furthermore, Ro52-mediated apoptosis was not influenced by p53 protein level in HeLa cells. Depleting Ro52 in HeLa cells caused Bcl-2, but not other Bcl-2 family molecules, to be upregulated. Taken together, our data showed that Ro52 is a universal proapoptotic molecule, and that its proapoptotic effect does not depend on p53, but is exerted through negative regulation of the anti-apoptotic protein Bcl-2. These findings shed light on a new physiological role for Ro52 that is important to intracellular immunity.

1. Introduction

SS-A/Ro52 (TRIM21) (Ro52), a 52-kDa ribonucleoprotein, is targeted by autoantibodies in Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) [1–3]. Although the mechanism by which anti-Ro52 autoantibodies are generated is not fully understood, there is evidence to suggest that Ro52 is expressed within apoptotic blebs during apoptosis evoked by stimuli such as ultraviolet-B (UV-B), viral infection, and TNF-α, among others [4]. On the other hand, we recently reported an alternative mechanism by which oxidative stress caused by UV-B and an oxidant, diamide, induces Ro52 to appear on keratinocytes without inducing apoptosis [5]. Nobuhara et al. reported that, in contrast to UV-B and diamide, H_2O_2 induces Ro52 translocation from the cytoplasm to the nucleus, suggesting that SS-A/Ro52 may serve as an oxidative stress-sensitive signaling molecule [6].

The Ro52 protein belongs to the rapidly growing RING/B-box/coiled-coil (RBCC) family, also called the TRIM (tripartite motif) family, which appears to carry out highly diverse functions within cells [7–9]. The Ro52 RBCC region consists of an N-terminal Zn^{2+} binding region containing a RING finger and a B-box, followed by a coiled-coil region and a putative leucine zipper domain. In many TRIM proteins, including Ro52, the RING domain has E3 ubiquitin ligase activity that can mediate autoubiquitylation and – less often – sumoylation or ISGylation of the TRIM protein itself along with other proteins [10]. TRIM proteins, functioning downstream of IFN and of pathogen-recognition receptors, modulate innate immune responses that activate IFN-regulatory factor 3 (IRF3), IRF7 and nuclear factor-κB (NF-κB) to fight bacterial and viral infections [4].

Ro52 was recently shown to have a physiological role as a high affinity immunoglobulin (Ig) receptor [11,12]. Mallery et al. reported that in HeLa cells exposed to antibody–adenovirus complexes, Ro52 rapidly bound the antibodies and targeted the complexes to proteasomes, possibly exerting its role as an E3 ubiquitin ligase before any virus-induced cytopathic effect was triggered [12]. This reveals a novel mechanism of antibody-mediated intracellular immunity. These data clearly demonstrate that antibodies bound to their antigens can penetrate cells, and that an intracellular pathogen destruction mechanism involving Ro52 exists as an adaptive immune response. On the other hand, *in vitro* studies have shown that Ro52 might downregulate immune responses by targeting interferon-responsive factors such as IRF3, IRF7, and IRF8 [13–15] and by interfering with the production of proinflammatory cytokines and activation of NF-κB by ubiquitinating IKKβ [16,17]. Furthermore, evidence that Ro52

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modulates the immune response is seen in Ro52-knockout mice, which develop SLE-like systemic autoimmunity [18].

Ro52 functions as a ubiquitin E3 ligase, an IgG receptor, and an autoantigen in autoimmune diseases. In this study, we describe a novel function of Ro52 that offers new insight into its role in immune reactions.

2. Materials and methods

2.1. Reagents and antibodies

Intron-A (IFNα-2b) was obtained from Merck (Whitehouse Station, NJ, USA), IFN-γ from R&D Systems (Minneapolis, MN, USA), and anti-Fas/CD95 antibody (Ab) from Medical and Biological Laboratories (Nagoya, Japan). Anti-Ro52, anti-p53, anti-Bcl-2, anti-Bcl-x_L, and anti-Bax antibodies (Abs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl2A1 Ab was obtained from Abcam (Cambridge, UK), diamide and etoposide from Sigma Aldrich (St. Louis, MO, USA), and NucBuster™ Protein Extraction Kits from Novagen (Madison, WI, USA). Negative control scrambled siRNA and siRNA for human TRIM21 (SS-A/Ro52) and p53 were purchased from Ambion (Austin, TX, USA). Target sequences of the TRIM 21 and p53 siRNA were 5′-AAGAGAGAUUUGAUAGUU-Att-3′ and 5′-GAAAUUUGCGUGUGGAGUAtt-3′, respectively. Nucleotide sequences of negative control scramble siRNA are not provided by Ambion (catalog number 4457287).

2.2. Cell culture and treatment

HeLa, SKOV-3, and MCF-7 cells were purchased from American Type Culture Collection, Japanese Collection of Research Bioresources, and Health Protection Agency (HPA) Culture Collections, respectively. All cell lines were maintained in DMEM with GlutaMAX (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and kept at 37 °C in a humidified incubator containing 5% CO_2 atmosphere.

For experiments with H_2O_2, diamide, IFN-α and etoposide, cells were incubated for 24 h with culture media containing 200 μM H_2O_2, 400 μM diamide, 3000 μM IFN-α or 50 μM etoposide. For experiments with γ-irradiation, the cells were washed, replaced with phosphate-buffered saline (PBS) free of any photoactive compounds, and exposed to 15 Gy γ-irradiation. The cells were then immediately reconstituted with complete medium and incubated for 24 h.

For experiments with IFN-γ and anti-Fas Ab, cells were first incubated for 24 h in culture media containing 50 ng/ml IFN-γ, and then incubated for 3 h with medium containing 250 ng/ml anti-Fas antibody to induce apoptosis.

2.3. siRNA transfection

Transfection with siRNA was performed with Lipofectamine RNAi-MAX (Invitrogen) according to the manufacturer's instructions. Briefly, HeLa cells (2.5×10^5) were transfected on 6-well plates or Lab-Tek chamber slides (Nalge Nunc, Taastrupgaardsvej, Denmark) in the antibiotic-free medium, with 6 nM TRIM21 siRNA or scramble control siRNA. In a parallel experiment, HeLa cells were transfected with 6 nM human p53 siRNA or a non-specific control siRNA. Transfected cells were subjected to further experiments 48 h after transfection.

2.4. Western blot analysis

Western blot analysis was performed in a standard method. Cytoplasmic and nuclear extracts were separated using the NucBuster™ Protein Extraction Kit. Samples containing 30–70 μg protein were subjected to SDS–PAGE using 5–20% Tris–glycine gels (PAGEL NPG-520L, ATTO, Tokyo, Japan). The separated proteins were transferred onto nitrocellulose membranes and blocked with 5% milk in TBS-T buffer. The blots were then incubated with the first Ab, triple-washed in TBS-T, and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Invitrogen) for 30 min at room temperature. Finally, the blots were triple-washed in TBS-T and developed with West Dura (Pierce).

2.5. Annexin V analysis

Annexin V analysis was conducted using the Annexin V-FLUOS staining kit (Roche Applied Science, Penzberg, Germany). Briefly, the treated cells were incubated for 24 h, washed with PBS, harvested, and resuspended in 100 μL of incubation buffer. Cells were then stained with 2 μL each annexin V-FITC and propidium iodide (PI), and incubated for 15 min at room temperature in the dark. The stained cells were analyzed using a FACSCalibur flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA) and BD CellQuest Pro software. Cells that were negative for PI and positive for annexin V were scored and counted as apoptotic cells.

2.6. TUNEL assay

Terminal transferase dUTP nick end labeling (TUNEL) analysis was conducted using a commercial ApoAlert DNA Fragmentation Assay kit (Clontech, Mountain View, CA, USA). A Carl Zeiss Pascal confocal laser scanning microscope (LSM5 Pascal Ver.3.0, Carl Zeiss, Oberkochen, Germany) equipped with lasers emitting at 496 and 568 nm was used to examine the cells. At least 500 cells were examined. Each sample was run in duplicate, and experiments were repeated at least three times.

2.7. Quantitative real time-PCR (QT-PCR)

Total cellular RNA was isolated with RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified RNA (1 μg) was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). Gene transcripts were quantified by real-time PCR using the QuantiTect SYBR Green PCR Kit (Qiagen). Amplification reactions and data acquisition and analyses were performed with the ABI Prism 7900 HT (Applied Biosystems, Foster city, CA, USA). Primer pairs used in the reactions were purchased from Qiagen; the catalogue numbers were QT00025011 for the Bcl-2 primers, and QT01658692t for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All values were normalized to the level of GAPDH mRNA and presented as the fold induction over control cells.

2.8. Statistical analysis

The data are presented as mean ± SD. Statistical analysis was conducted using Student's unpaired *t*-test. Results were considered to be statistically significant compared with the control for $P < 0.05$.

3. Results

3.1. $Ro52^{low}$ HeLa cells are resistant to stress-induced apoptosis

To determine whether Ro52 plays a role in stress-induced apoptosis, we first used flow cytometry to quantitate apoptosis following various types of apoptotic stimulation in HeLa cells transfected with either scramble or Ro52-specific siRNA. As shown in Fig. 1(A and B), there was a significant decrease in annexin V-positive cells among

Ro52^{low} as compared to wild-type HeLa cells, whether untreated or subjected to apoptotic stimuli, which included a 24-h incubation with one of the following, H_2O_2, diamide, IFN-α, IFN-γ and anti-Fas Ab, or etoposide, or exposure to γ-irradiation. These results suggest that Ro52 acts as a proapoptotic molecule under several types of apoptotic stimulation. As there were fewer apoptotic cells among Ro52^{low} HeLa cells than among control HeLa cells, Ro52 may have a proapoptotic role even in normal conditions. We also conducted dose-dependent experiments in both Ro52^{low} and control HeLa cells cultured in the presence of H_2O_2 or diamide for 24 h. The number of annexin V-stained cells decreased in Ro52^{low} HeLa cells incubated with either a low or a high concentration of H_2O_2; however, the apoptotic fraction decreased only with higher concentrations of diamide (400 μM) (Fig. 1C). To confirm this phenomenon, we used a TUNEL assay to analyze apoptosis-associated DNA fragmentation in the stimulated Ro52^{low} HeLa cells. Exposing cells to H_2O_2, diamide, IFN-α, IFN-γ and anti-Fas Ab, etoposide, or γ-irradiation resulted in nuclear DNA fragmentation, which is characteristic of apoptotic changes. Consistent with these results, Ro52^{low} HeLa cells displayed less apoptotic morphology than control HeLa cells after exposure to proapoptotic stimuli (Fig. 1D).

3.2. Ro52 is upregulated and translocated to the nucleus by IFN-α, etoposide, or IFN-γ and anti-Fas Ab, but not by H_2O_2, diamide, or γ-irradiation

To investigate Ro52's role under stress conditions, we first determined whether Ro52 expression in HeLa cells was affected by cytokines or apoptotic stimuli. As shown in Fig. 2, Western blot analysis revealed that while Ro52 levels did not change noticeably in HeLa cells stimulated with H_2O_2, diamide, or γ-irradiation, they increased significantly 24 h after stimulation with IFN-α, IFN-γ and anti-Fas Ab, or etoposide. When we analyzed cytoplasmic and nuclear fractions separately, we found that Ro52 accumulated in the nucleus of HeLa cells stimulated with IFN-α, IFN-γ and anti-Fas Ab, or etoposide, but not in those stimulated with H_2O_2, diamide, or γ-irradiation, although these latter three stimuli induced similar levels of apoptosis. These findings indicate that Ro52 nuclear translocation occurs in response to specific stimuli. This,

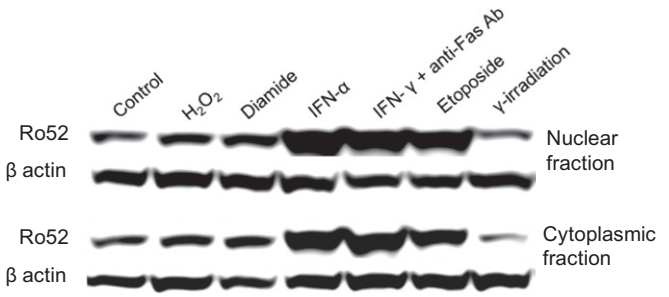

Fig. 2. IFN-α, IFN-γ and anti-Fas Ab, and etoposide induce Ro52 upregulation and nuclear translocation. Nuclear and cytoplasmic fractions were extracted from HeLa cells, which were cultured in the presence of H_2O_2, diamide, IFN-α, IFN-γ and anti-Fas Ab, etoposide, or γ-irradiation. Ro52 expression and nuclear translocation were determined by immunoblotting with anti-Ro52 Ab.

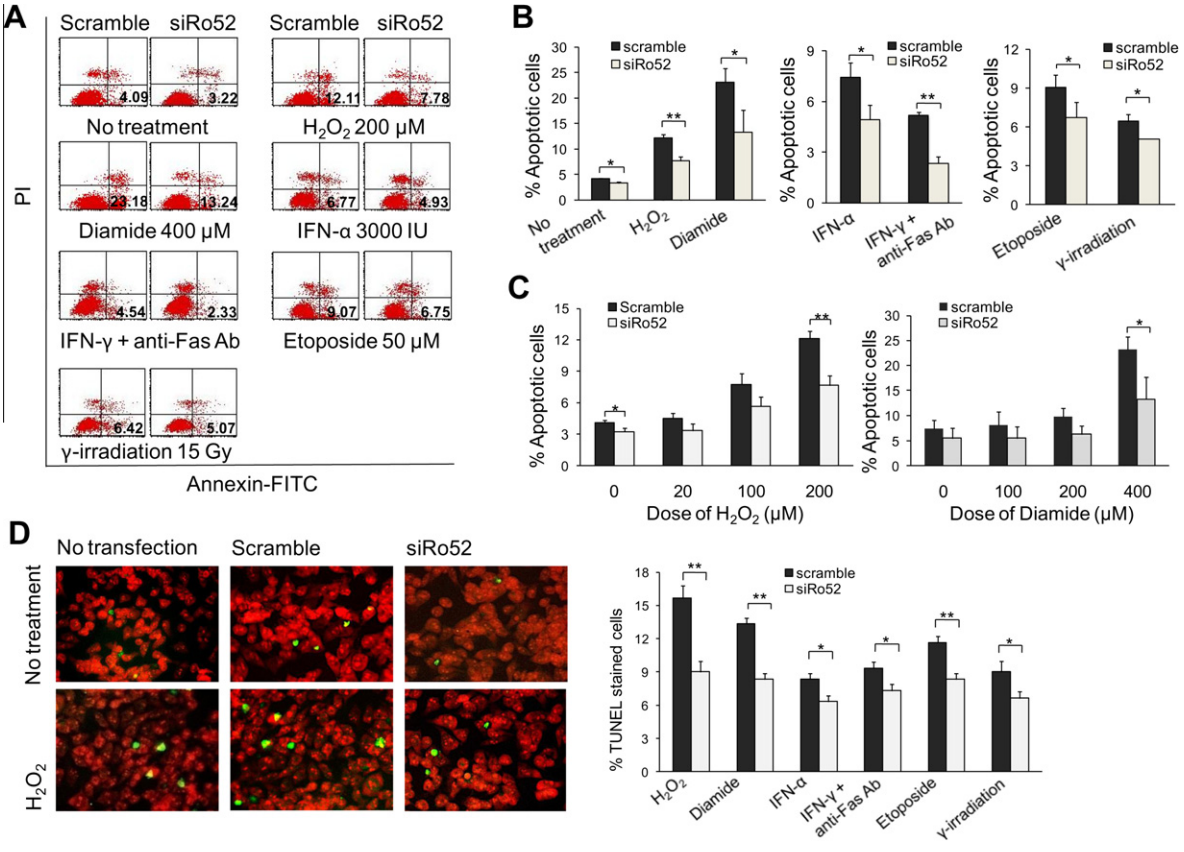

Fig. 1. Ro52^{low} HeLa cells are resistant to stress-induced apoptosis. (A and B) HeLa cells transfected with scramble siRNA or Ro52-specific siRNA were cultured in the presence of H_2O_2, diamide, IFN-α, IFN-γ and anti-Fas Ab, etoposide, or were exposed to γ-irradiation. Cells were harvested at 24 h after stimulation, and apoptosis was measured by flow cytometry with Annexin V-FITC and PI counterstaining. Triplicate experiments were performed. (C) Ro52^{low} HeLa and control cells were stimulated with H_2O_2 and diamide at the indicated doses. Apoptosis was analyzed by flow cytometry. (D) TUNEL assays were performed in Ro52^{low} HeLa and control HeLa cells treated with apoptotic stimuli. Apoptotic cells showed green nuclear fluorescence and were calculated as the percentage of TUNEL-positive cells. Data are represented as mean ± SD of triplicates and are representative of three independent experiments. $^{*}P < 0.05$. $^{**}P < 0.01$.

together with the data in Fig. 1, indicates that the Ro52-mediated apoptotic process occurs without regard to Ro52 nuclear translocation, and that Ro52 may be a universal proapoptotic molecule.

3.3. Ro52-mediated apoptosis is independent of p53

Since etoposide, a DNA topoisomerase II inhibitor which has already been known to cause apoptosis in a p53-dependent manner [19], caused the upregulation and translocation of Ro52 into the nucleus in our experiment, we examined whether p53 is involved as an upstream molecule inducing Ro52-mediated apoptosis or not. We analyzed Ro52 expression in HeLa cells transfected with either p53-specific or control siRNA. Ro52 levels did not differ between $p53^{low}$ or control HeLa cells (Fig. 3A). Ro52 was upregulated in both $p53^{low}$ and control HeLa cells incubated with IFN-α, etoposide, or IFN-γ and anti-Fas Ab for 24 h. The same experiments performed in the p53-null SKOV-3 and p53-germline MCF-7 cells showed the same effects on Ro52: it was upregulated in the presence of IFN-α, IFN-γ and anti-Fas Ab, or etoposide (Supplementary Fig. 1). Thus, Ro52-mediated apoptosis is not regulated by p53.

To investigate the possibility that Ro52 might regulate p53 expression and exert a proapoptotic effect through a p53-mediated pathway, we conducted Western blot analyses of p53 expressed in $Ro52^{low}$ HeLa cells treated with various types of apoptotic stimuli. As shown in Fig. 3B, there was no difference in p53 expression between $Ro52^{low}$ and control HeLa cells. In H_2O_2 dose-dependent stimulation experiments, p53 levels did not differ between $Ro52^{low}$ and control HeLa cells (Fig. 3C). Therefore, Ro52 did not influence to p53 expression. Taken together, the proapoptotic effect of Ro52 uses a mechanism that is independent of the p53-mediated pathway.

3.4. Ro52 selectively regulates Bcl-2 expression

As Bcl-2 family molecules are important in regulating apoptosis, we examined the effect of knocking down Ro52 on the expression of Bcl-2 family molecules in HeLa cells. Western blot analyses revealed that Bcl-2 was upregulated by Ro52 knockdown in the presence of apoptotic stimuli, but Bax, Bcl2-A1 (Bfl-1), and Bcl-x_L were not (Fig. 4A). Notably, Bcl-2 levels in $Ro52^{low}$ cells were higher than in control HeLa cells even in the absence of apoptotic stimulation, which parallels the finding of low numbers of apoptotic cells in non-treated $Ro52^{low}$ HeLa cells. In addition, QT-PCR confirmed Bcl-2 mRNA upregulation after apoptotic stimulation in $Ro52^{low}$ HeLa cells, which indicates that Ro52 regulates the Bcl-2 protein at the transcriptional level (Fig. 4B). These results suggest that Ro52 selectively downregulates Bcl-2 mRNA among Bcl-2 family molecules, and that the Ro52 protein may exert its proapoptotic effect by downregulating the expression of anti-apoptotic protein Bcl-2.

4. Discussion

We have presented Ro52 in a new role as a proapoptotic molecule. We showed that Ro52 suppressed Bcl-2 expression at the level of mRNA transcription. As IFN-α induces Ro52 expression, Ro52 may induce apoptosis in host cells by negatively regulating the Bcl-2-mediated anti-apoptotic system in viral infection. Since cytotoxic T cells act as a main effecter against viral infection by killing infected host cells, it is intriguing to speculate that Ro52 might act as an effecter molecule to kill host cells from the inside. Interestingly, $Ro52^{low}$ HeLa cells had fewer apoptotic cells and higher Bcl-2 levels than were found in control cells even in the absence of apoptotic stimulation. These results suggest that Ro52's apoptotic effect may be a fundamental mechanism for maintaining cellular homeostasis. In addition, the apoptosis triggered by etoposide was attenuated by downregulation of Ro52. Since etoposide is known to cause apoptosis in a p53-dependent pathway, our results suggest that etoposide may cause apoptosis in both p53-dependent and -independent pathways. Therefore, further investigation is required to reveal the unknown roles of Ro52 in apoptotic pathways including how Ro52 regulates the transcription of Bcl-2.

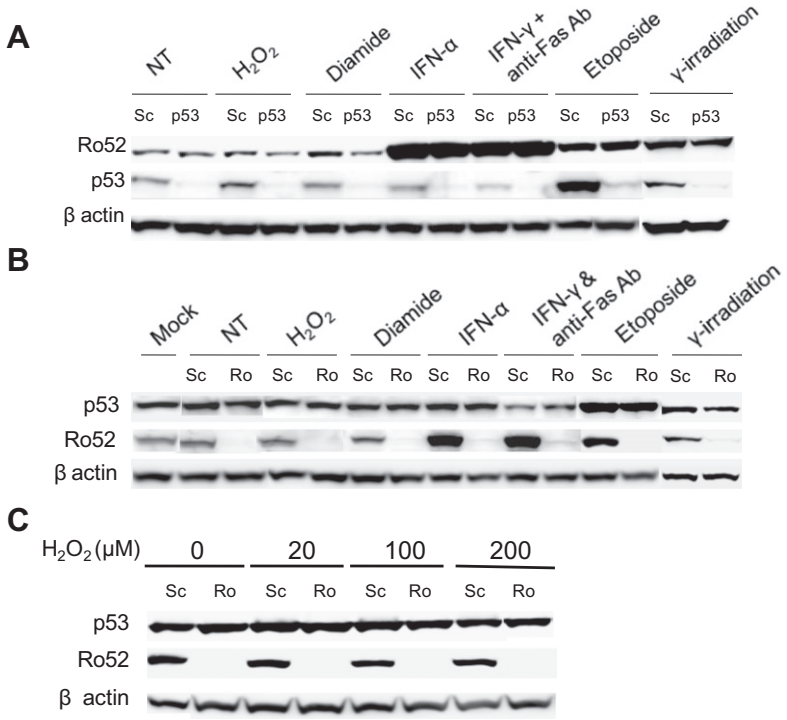

Fig. 3. Ro52-mediated apoptosis is independent of p53. (A) Both $p53^{low}$ and control HeLa cells were treated with H_2O_2, diamide, IFN-α, IFN-γ with anti-Fas antibody, etoposide, or γ-irradiation. Cell lysates were collected 24 h later and immunoblotted to determine the Ro52 levels. (B) $Ro52^{low}$ and control HeLa cells were stimulated with either H_2O_2, diamide, IFN-α, IFN-γ and anti-Fas Ab, etoposide, or γ-irradiation. After 24 h, cell lysates were subjected to immunoblotting with anti-p53 Ab. (C) $Ro52^{low}$ and control HeLa cells were incubated with H_2O_2 at the indicated doses. Twenty-four hours after stimulation, cell lysates were subjected to immunoblotting with anti-p53 Ab.

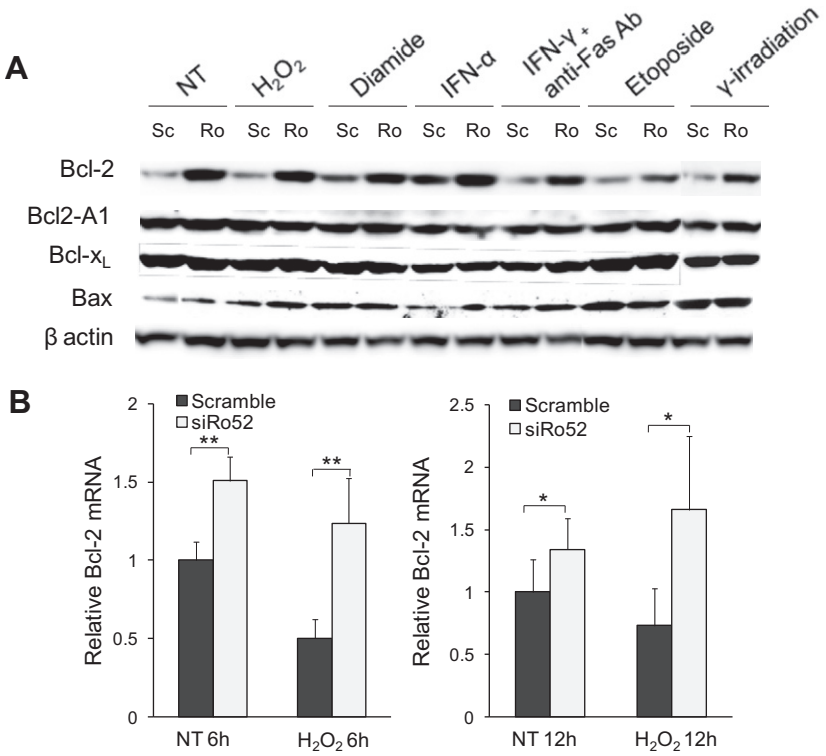

Fig. 4. Bcl-2 is overexpressed in Ro52^{low} HeLa cells. (A) Ro52^{low} and control HeLa cells were stimulated with either H_2O_2, diamide, IFN-α, IFN-γ and anti-Fas Ab, etoposide, or γ-irradiation. After 24 h, cell lysates were collected and the levels of Bcl-2, Bcl2-A1, Bcl-x_L, and Bax were determined by immunoblotting with specific Abs. (B) QT-PCR for Bcl-2 mRNA expression. Total RNA was extracted from Ro52^{low} and control HeLa cells in the presence or absence of H_2O_2 at the indicated time points. The results were normalized to GAPDH. Bars represent mean ± SD from triplicate quantitation. Similar results were obtained in two additional experiments. $^{*}P < 0.05$. $^{**}P < 0.01$.

Strandberg et al. reported that Ro52 nuclear translocation precedes apoptosis upon IFN-α stimulation in HeLa cells [20]. We previously reported that Ro52 nuclear translocation occurred in HaCaT cells treated with 100 mM H_2O_2, a concentration high enough to cause apoptosis in 10% of the cells [5,6]. However, our results in HeLa cells showed that apoptosis occurred after stimulation by H_2O_2 (200 mM), diamide, or γ-irradiation even when Ro52 nuclear accumulation was not detected. These results suggest that Ro52 nuclear accumulation after IFN-α or H_2O_2 treatment might not be directly linked to apoptosis. Our results also showed that certain apoptotic stimuli, such as etoposide or IFN-γ and anti-Fas Ab, caused Ro52 nuclear translocation. Therefore, it is too early to describe a simple mechanism that explains the role of nuclear Ro52 accumulation in apoptosis.

Espinosa et al. reported that Ro52 levels are significantly increased in the peripheral blood mononuclear cells (PBMC) of patients with SLE and SS [21]. When Ro52 was overexpressed in a mouse B cell line, steady-state growth decreased and cell death increased after activation via the CD40 pathway. Reducing Ro52 expression restored cell viability, clearly demonstrating Ro52's role in activation-induced cell death [21]. These findings suggest that increased Ro52 expression may be directly involved in the reduced cellular proliferation and increased apoptotic cell death observed in PBMCs from patients with SLE and SS. These researchers speculated that Ro52 accumulation may contribute to the autoantigenic load and the induction of autoimmune B and T cell responses. Our data using HeLa cells, an epithelial cell-derived line, also showed that Ro52 accumulated in response to various stimuli, including IFN-α, IFN-γ and anti-Fas Ab, the cytotoxic drug etoposide. These data strongly suggest that Ro52 has an important role in connecting various extracellular stimuli and the intracellular apoptotic pathway.

Since the first description of anti-ribonucleoprotein Ab entering into Tγ lymphocytes [22], other cell-penetrating Abs have been found, including anti-dsDNA Abs, anti-nuclear Abs in kidney and liver cells, and anti-SS-A/Ro and -SS-B/La Abs in salivary gland epithelial cells [23]. Abs may penetrate cells via membrane Fc receptor (FcR)-mediated intracellular uptake, but cells lacking FcRs also take up Abs [24]. Ro52 is unique as an autoantibody. Mallery et al. reported that Ro52 acts as a cytosolic IgG receptor in cells, binding to antibodies with a higher affinity than any other IgG receptors in the human body [25]. Ro52 is rapidly recruited to an incoming Ab-bound virus and targets it to the proteasome via its E3 ubiquitin ligase activity [25]. Then, the question is what role the anti-Ro52 Ab plays in autoimmune disease? Once an anti-Ro52 Ab enters the cell by any mechanism, it could block Ro52's various biochemical roles – including its E3 ubiquitin ligase activity, its IgG Fc receptor binding, and its nuclear translocation. If an anti-Ro52 Ab attacked the Ro52-IgG complex, the intracellular adaptive immunity mediated by the complex would probably suffer. A recent report by Espinosa et al. supports the idea. They showed that anti-Ro52 autoantibodies inhibited the E3 ligase activity of Ro52 by blocking the E2/E3 interaction between Ro52 and UBE2E1 [26]. Therefore, some anti-Ro52 Abs might block Ro52's ability to ubiquitinate IRF family molecules, resulting in sustained activation of interferon-mediated pathway and high production of type I interferon and inflammatory cytokines. On the other hand, such Abs could also lead to defects in neutralizing other simultaneously existing autoantibodies, such as anti-Sm and anti-RNP Abs, which are found in SLE, or anti-SS-A/Ro and anti-SS-B/La Abs, found in cases of SS. The resulting accumulation of autoantigen–autoantibody complexes might exacerbate autoimmune pathogenesis. McNab et al. speculated that, although Ro52 at physiological expression levels may rapidly neutralize intracellular autoantibodies, these autoantibodies may be more pathogenic if Ro52 expression is low [21]. Our data suggest that the upregulation of Ro52 may be canceled by the existence of intracellular anti-Ro52 Ab upon various stress conditions in SS or SLE patients. These ideas are intriguing, but need to be examined in further studies.

In summary, Ro52 function is connected to various environmental stimuli and is important in inducing apoptosis by repressing Bcl-2 protein production. Since Ro52's proapoptotic effect is independent of p53, Ro52 may be another critical gatekeeper of cell damage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.010.

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