

Nuclear receptor subfamily 4, group A, member 1 inhibits extrinsic apoptosis and reduces caspase-8 activity in H₂O₂-induced human HUC-F2 fibroblasts

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Objective: Apoptosis is characterized by distinct morphological and biochemical changes that occur upon activation of a family of serine proteases known as caspases. Reactive oxygen species (ROS) induce apoptosis in many cell systems. Nuclear receptor subfamily 4, group A, member 1 (NR4A1) has been shown to induce apoptosis in a number of cell lineages, but can also paradoxically act as a death inhibitory factor. In the current study, we focused on the potential role of NR4A1 in hydrogen peroxide (H₂O₂)-induced apoptosis of normal human umbilical cord fibroblast (HUC-F2) cells.

Methods: Growth of HUC-F2 cells treated with H₂O₂ was measured by MTT assay. Analysis of gene expression was performed with a STEP ONE PLUS Real Time PCR system. Inactivation of NR4A1 was treated with siRNA. Apoptosis was measured by Beckman Coulter flow cytometer after inhibition of NR4A1 with siRNA and H₂O₂ treatment. Caspase -3, -8 and -9 was measured by caspase assay kit.

Results: H₂O₂ treatment led to enhanced NR4A1 expression. Moreover inhibition of NR4A1 with specific siRNA in HUC-F2 cells triggered an increase in apoptosis and caspase-8 and -3 activities following the addition of H₂O₂.

Discussion: Our results collectively suggest that NR4A1 is a regulator that inhibits extrinsic apoptosis in HUC-F2 cells during oxidative stress through reduction of caspase-8 and -3 activities.

Keywords: NR4A1, Apoptosis, Caspase-8, Oxidative stress

Introduction

Oxygen is both essential for aerobic life, being the terminal electron acceptor in respiration, and hazardous, because of the potency of reactive oxygen species (ROS). ROS include the superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and singlet oxygen, which are produced by neutrophils and macrophages¹ in the human body. At excess concentrations or in the wrong location, ROS can damage proteins, carbohydrates, polyunsaturated fatty acids, and DNA, and lead to a variety of degenerative processes and diseases.² Apoptosis, a programmed cell death process characterized by distinct morphological and biochemical changes that take place upon the activation of a family of serine proteases known as caspases,³ plays important roles in the development, homeostasis, and anticancer defence of multicellular organisms.⁴⁻⁶ ROS have been shown to induce apoptosis in several different cell systems.⁷

Nuclear receptor subfamily 4, group A, member 1 (NR4A1, also known as Nur77 or TR3) is widely expressed but primarily detected in the thymus, osteoblast, liver, and pituitary gland.^{8,9} NR4A1, a member of an orphan nuclear receptor superfamily, was originally recognized for its role in the regulation of cell survival and differentiation.^{10,11} Expression of NR4A1 is induced by multiple stimuli in different cell types. NR4A1 has been shown to trigger apoptosis in a number of cell lineages exposed to pro-apoptotic stimuli by directly targeting mitochondria and inducing cytochrome-c release.¹²⁻¹⁵ Paradoxically, NR4A1 is also reported to act as a death inhibitory factor, blocking cell death induced by ceramide¹⁶ or tumour necrosis factor (TNF).¹⁷ The finding to date support important roles of these receptors in cell transformation and tumorigenicity via both their anti-apoptotic and pro-apoptotic functions. In particular, the recent identification of a functional ligand (1,1-bis (3'-indolyl)-1-(p-substituted phenyl) methane containing trifluoromethyl, hydrogen, and methoxy substituents) for NR4A1 suggests that members of

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this superfamily could potentially serve as drug targets for disease indications, such as cancer.¹⁸

Although apoptotic and carcinogenic roles of NR4A1 have been discussed with respect to its potential as a therapeutic target in cancer cells, limited reports of NR4A1 activity in normal human cell of human umbilical cord fibroblast (HUC-F2) are documented. Therefore, in the current study, we focused on the mechanisms that determine whether NR4A1 exerts pro- or anti-apoptotic activity in normal human cells. In our experiments, inhibition of NR4A1 with siRNA led to increased apoptosis and caspase-8 activity in H₂O₂-treated human fibroblast HUC-F2 apoptotic cells. Based on the collective finding, we propose NR4A1 acts as a regulator of HUC-F2 cell death under oxidative stress conditions and prevents extrinsic apoptosis through reducing caspase-8 activity.

Experimental methods

Cell culture

HUC-F2 cells were obtained from Riken BioResource Center Cell Bank (Ibaraki, Japan). Cells were cultured in Alpha-Modified Eagle's Medium (α -MEM) (Cellgro, Manassas, VA, USA) containing 10% foetal bovine serum (FBS) (MP Biomedicals Inc., Solon, OH, USA), 150 IU/ml penicillin (Meiji Seika Kaisha, Tokyo, Japan), and 100 mg/ml streptomycin (Meiji Seika Kaisha) in humidified air at 37°C with 5% CO₂.

Cytotoxicity assays

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) colorimetric assay was performed in 96-well plates.¹⁹ Briefly, HUC-F2 cells were seeded in 96-well plates at a concentration of 6.0×10^3 cells/well, and incubated at 37°C with 5% CO₂. The medium was removed and cells in each well incubated with 50 μ l MTT solution (5 mg/ml) for 3 hours at 37°C with 5% CO₂. The MTT solution was discarded and 50 μ l dimethyl sulphoxide added to dissolve insoluble formazan crystal. Absorbance was measured at 550 nm.

Real-time RT-PCR

Total RNA was extracted from HUC-F2 cells, and cDNA was synthesized as described earlier.²⁰ Briefly, total RNA was extracted using Isogen reagent (Nippon gene, Toyama, Japan), and first-strand cDNA, generated from DNase I-treated total RNA (4 μ g) with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Analysis of gene expression was performed with a StepOnePlus Real-Time PCR system (Applied Biosystems Japan, Tokyo, Japan) using SYBR Green PCR Master Mix (Applied Biosystems Japan) and the following primers: NR4A1 sense,

5'-CACAGCTTGCTTGTCGATGTC-3' and anti-sense, 5'-ATGCCGGTCGGTGATGAG-3'; NR4A3 sense, 5'-GCATCCCCCATGGTTCAG-3' and anti-sense, 5'-TCAGTTGGTGCTCCCCTTGT-3'; β -actin sense, 5'-CTTCTACAATGAGCTGCGTG-3' and antisense 5'-TCATGAGGTAGTCAGTCAGG-3'; and GAPDH sense 5'-TGCCTCCTGCACCACCAACT-3' and antisense, 5'-AGTAGAGGCAGGGATGATGT-3'. PCR conditions as follows: first incubation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. Normalization of data was achieved by quantitating the cycle time at an arbitrary fluorescence intensity in the linear exponential phase using StepOnePlus Real-Time system software (Applied Biosystems Japan) by calculating the ratio of the relative concentration of each enzyme relative to that of β -actin and GAPDH cDNA, respectively.

Inactivation of NR4A1 with siRNA

The siRNAs used for the experiment were obtained from Life Technology Japan (Tokyo, Japan). The siRNA sequences for NR4A1 were s6680 (GC AUUAUGGUGUCCGCACAtt), s6679 (CCACUUCUCCACACCUUGAtt), s6678 (GCACCUUC AUGGACGGCUAtt) (Silencer Select Pre-designed siRNA), 232748F11 (CCGGUUCUCUGGAGGU CAUCCGCAA), 232748G01 (GGUCCUGCACA GCUUGCUUGUCGA), and 232903D12 (CAGCAUUAUGGUGUCCGCACAUGUG) (Stealth RNAi). Negative control siRNAs were used according to the manufacturer's instructions. Lipofectamine 2000 was obtained from Life Technology Japan. For dilution of siRNA, Opti-Modified Eagle's Medium (Opti-MEM) I Reduced Serum Medium (GIBCO-Life Technology Japan) was used. Procedures for inactivation were performed using the manufacturer's protocol. Briefly, Lipofectamine 2000 was diluted with Opti-MEM I Reduced Serum Medium and equilibrated for 5 minutes at room temperature. The siRNA was mixed with Opti-MEM I Reduced Serum Medium, incubated for 20 minutes at room temperature, and added to the culture medium. The final concentrations of siRNA and Lipofectamine 2000 were 10 nM and 0.2%, respectively. HUC-F2 cells were cultured in α -MEM containing 10% FBS and 150 IU/ml to 100 μ g/ml penicillin to streptomycin in the presence of siRNA and Lipofectamine 2000, followed by incubation in 5% CO₂ for 48 hours.

Knockdown efficiency was determined using real-time RT-PCR. To determine off-target effects, we searched for homologous sequences using BLAST.

Apoptosis assay

Cellular apoptosis was detected with flow cytometry (FCM) on a Cell Lab Quanta SC MPL instrument (Beckman Coulter, Tokyo, Japan). Briefly, HUC-F2 cells (8×10^5 cells/ml) were seeded into five wells on

a six-well plate and treated with siRNA. After 48 hours, the medium was changed to α -MEM medium containing 200 μ M H_2O_2 , followed by incubation for a further 2 hours. Following treatment with H_2O_2 , cells were washed with PBS and stained with Annexin V-FITC (Beckman Coulter), according to the manufacturer's instructions. Control HUC-F2 cells (treated with si-NC or not exposed to H_2O_2) were subjected to the same protocol.

Caspase assay

Caspase activity was detected with the ApoAlert Caspase Assay Plate (Clontech, CA, USA), which contained fluorogenic substrates specific for caspases-3, -8, and -9 immobilized in the wells of a 96-well plate, according to the manufacturer's instructions. Briefly,

pelleted HUC-F2 cells (2×10^5 cells) were incubated with 50 μ l of 1 \times cell lysis buffer on ice for 10 minutes. Cell lysates were centrifuged at 15 000 rpm for 5 minutes at 4°C. Supernatant fractions were transferred to a 96-well plate pre-incubated with 50 μ l of 2 \times reaction buffer/dithiothreitol (DTT) mix at 37°C for 5 minutes. After incubation for 2 hours at 37°C, the release of 7-amino-4-methyl coumarin fluorogenic dye was detected using a microplate reader (excitation: 380 nm, emission: 460 nm) (BioTek Japan, Tokyo, Japan).

Statistical analysis

All data are presented as means \pm SEM. Statistical analysis was performed using the StatView (SAS Institute Inc. Cary, NC, USA). P values < 0.05 were

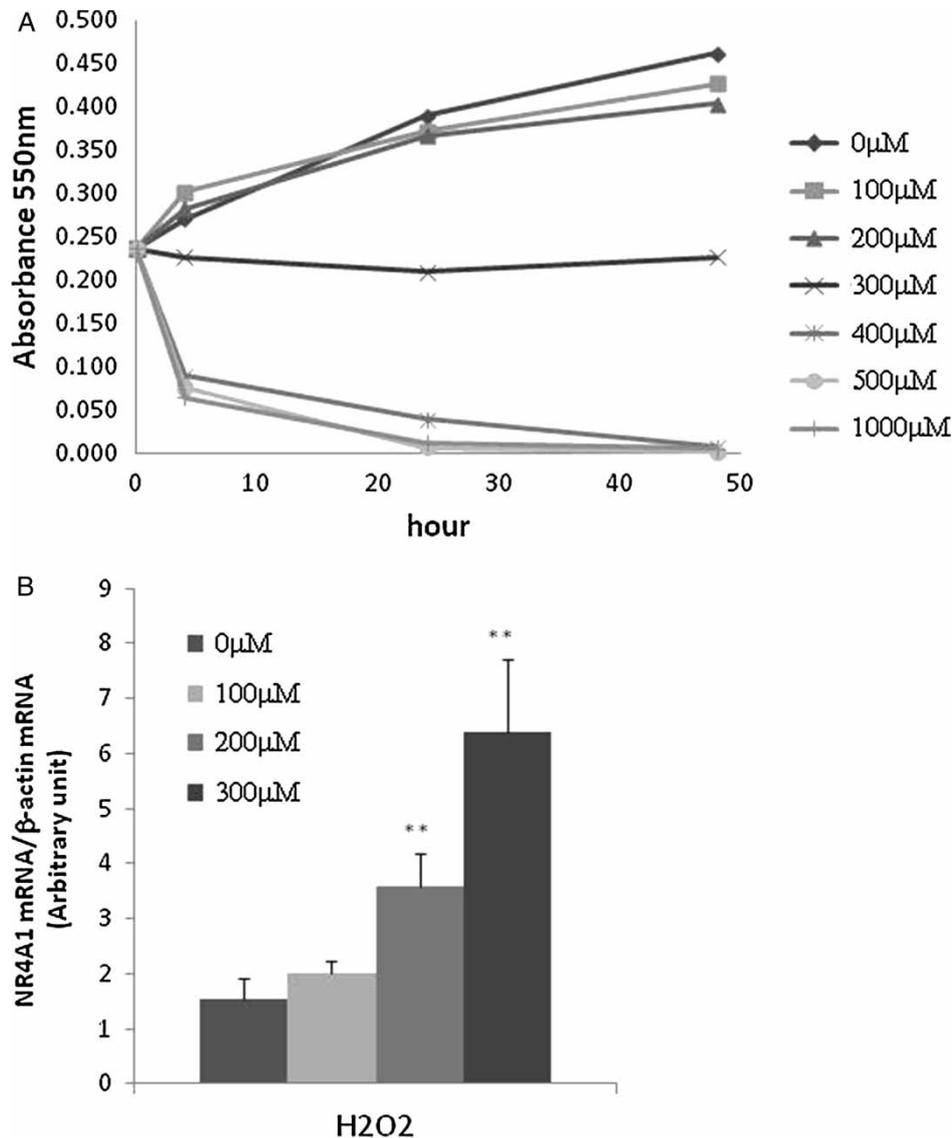


Figure 1 Effect of H_2O_2 on HUC-F2 cells. (A) HUC-F2 cells were treated with 0–1000 μ M H_2O_2 incubated in 5% CO_2 for 48 hours, and viability was assessed with the MTT colourimetric assay, as described in Experimental Methods. High concentrations of H_2O_2 inhibited HUC-F2 cell growth. (B) NR4A1 mRNA levels were determined with real-time PCR in HUC-F2 cells treated with 0–300 μ M H_2O_2 for 4 hours. Results are presented as mean \pm standard deviation of three samples in each treatment group. ** $P < 0.01$ versus control. Expression of NR4A1 was increased by H_2O_2 in a concentration-dependent manner. The differences between the groups were analyzed by ANOVA with Fisher's PLSD *post hoc* method.

considered statistically significant, as determined using ANOVA with Fisher's Protected Least Significant Difference (PLSD) *post hoc* method.

Results

H_2O_2 induces NR4A1 expression

We measured the growth of HUC-F2 cells treated with different concentrations of H_2O_2 for up to 48 hours with the MTT assay. After 6 hours, cells treated with 400 μ M H_2O_2 or more concentrations died (Fig. 1A). To determine the specific role of NR4A1 in H_2O_2 -mediated apoptosis, its expression patterns under

conditions of oxidative stress were measured in cells after 4 hours of treatment using real-time PCR. Our data showed that NR4A1 expression is enhanced by H_2O_2 in a concentration-dependent manner (Fig. 1B).

Efficiency of siRNA

The involvement of NR4A1 in cell death induced by oxidative stress was determined by transient transfection of HUC-F2 cells with NR4A1-specific siRNA (si-NR4A1). NR4A1 expression was significantly reduced upon transfection of cells with NR4A1-targeting siRNA, compared with control siRNA (si-NC), at 24, 48, and 72 hours when the siRNA sequence

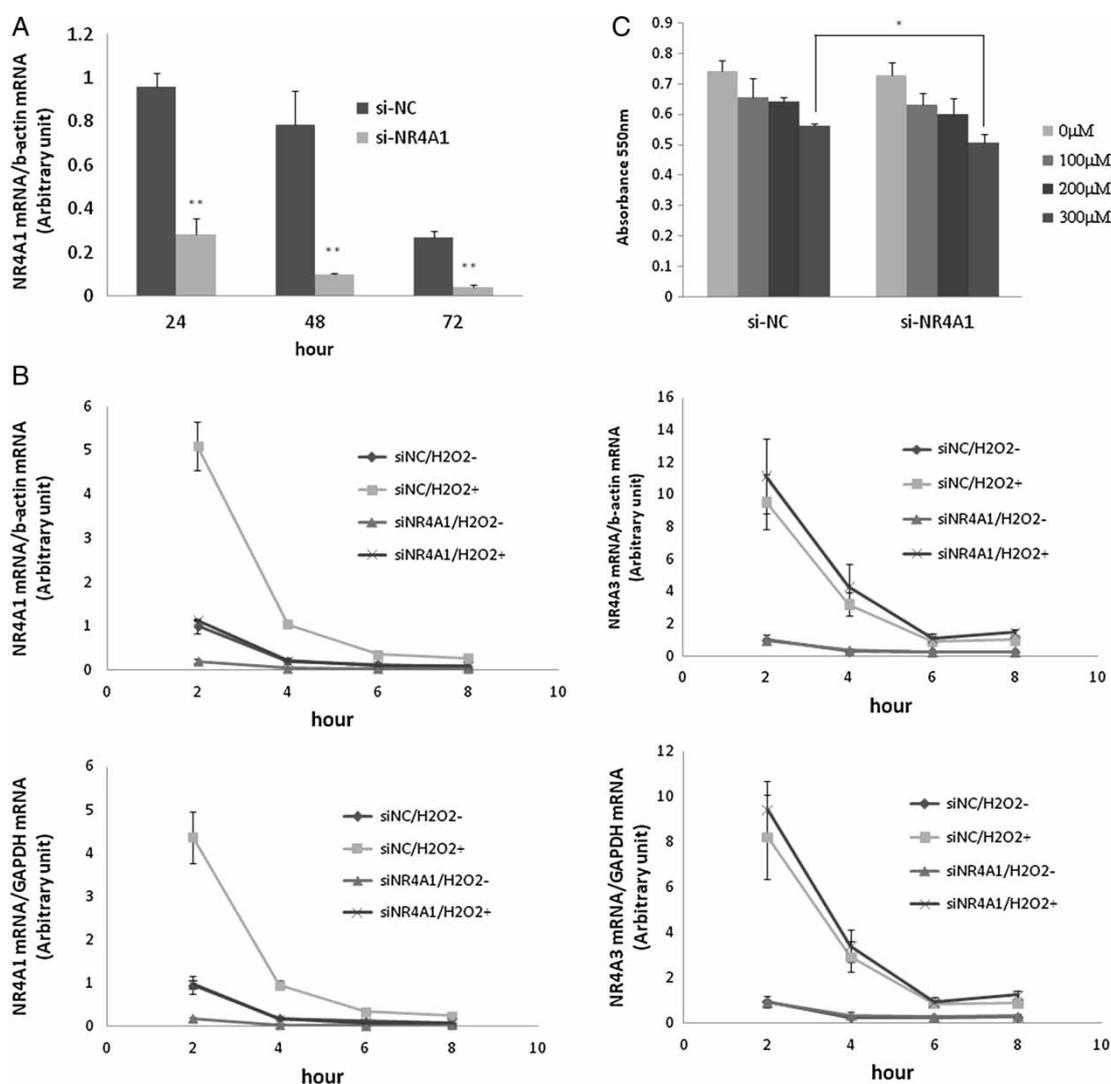


Figure 2 Effect of si-NR4A1 on mRNA expression and cell growth. (A) HUC-F2 cells were cultured with 10 nM siRNA and 0.2% Lipofectamine 2000 in α -MEM containing 10% fetal bovine serum and 150 IU/ml to 100 μ g/ml penicillin to streptomycin medium for 24, 48, and 72 hours in 5% CO_2 . Real-time PCR analysis showed that NR4A1 expression is reduced by 70.4, 87.2, and 83.7%, respectively. (B) HUC-F2 cells were transfected with si-NR4A1 for 48 hours and treated with 200 μ M H_2O_2 for 2–8 hours. Expression levels of NR4A1 and NR4A3 were determined using real-time PCR. Normalization of data was achieved by quantitating the cycle time at an arbitrary fluorescence intensity in the linear exponential phase using StepOnePlus Real-Time system software by calculating the ratio of the concentration of each enzyme relative to that of β -actin and GAPDH cDNA, respectively. (C) Effects of the H_2O_2 concentration on the HUC-F2 cells treated with si-NR4A1 were determined with the MTT assay. Specifically, HUC-F2 cells were transfected with si-NR4A1 or si-NC, incubated in 5% CO_2 for 48 hours, and treated with H_2O_2 . After 24 hours, cell viability was assessed with the MTT assay. Results are presented as mean \pm standard deviation of three samples in each treatment group. The differences between the groups were analyzed by ANOVA with Fisher's PLSD *post hoc* method. * $P < 0.05$ versus control; ** $P < 0.01$ versus control.

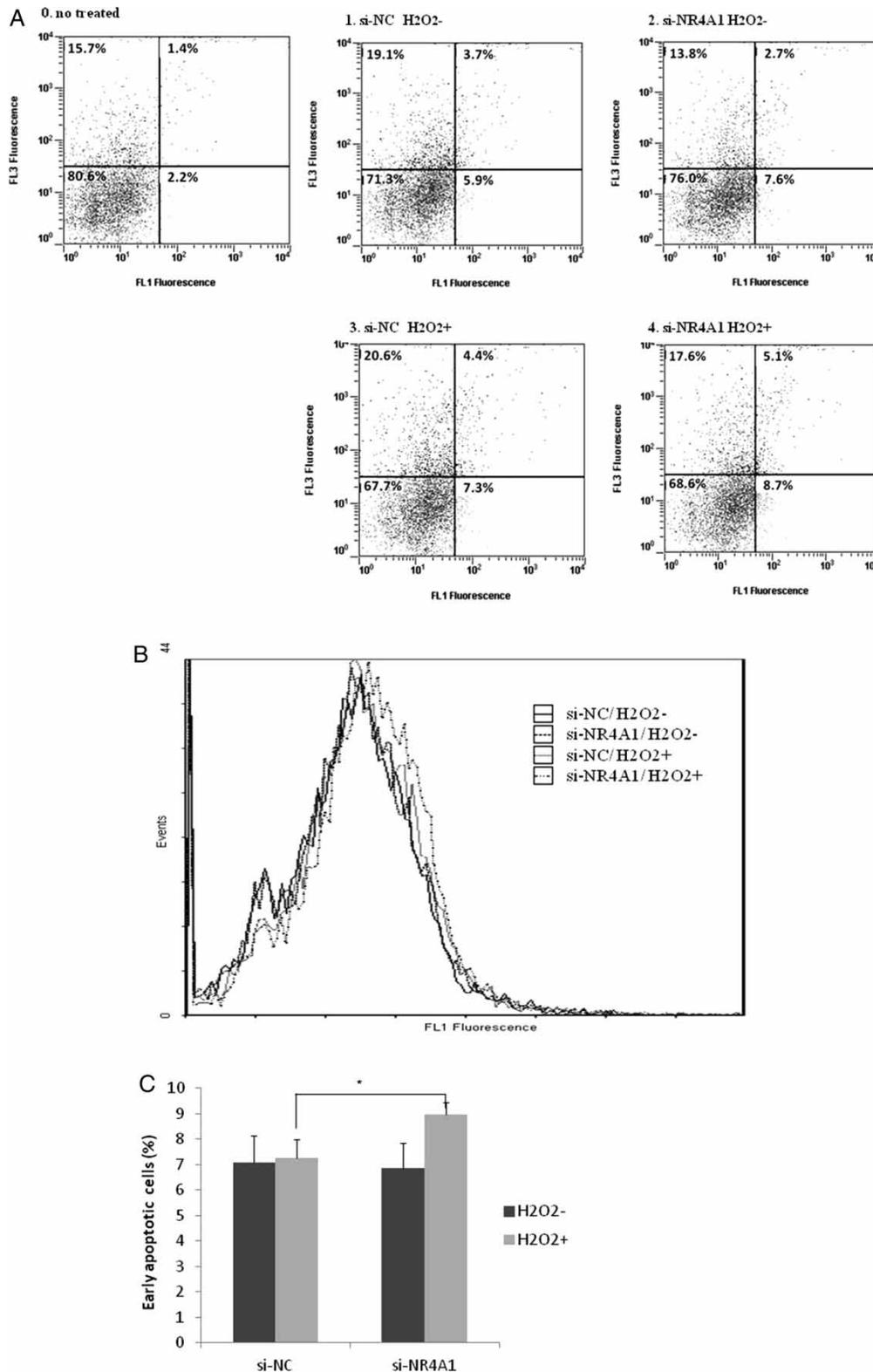


Figure 3 Determination of cell apoptosis using FCM. HUC-F2 cells transfected with si-NC or si-NR4A1 were left untreated or treated with H₂O₂ at a concentration of 200 μM for 2 hours, processed for annexin V/propidium iodide (PI) staining, and analyzed using Beckman Coulter FCM. (A) The apoptotic region was determined in relation to normal untreated HUC-F2 cells. Viable cells are located in the bottom left corner (negative for annexin V-FITC and PI), as observed for most normal HUC-F2 cells (A0). Early apoptotic cells are present in the bottom right corner (annexin V-FITC positive). Late apoptotic cells showing signs of progressive cellular membrane and nuclear damage are in the upper right corner (double positive), and the four divisions were fixed with this gating. Cells transfected with si-NC without H₂O₂ (A1), si-NR4A1 without H₂O₂ (A2), si-NC with 200 μM H₂O₂ (A3), and si-NR4A1 with 200 μM H₂O₂ (A4) were analyzed. (B) Distribution of annexin V-positive cells. Annexin V-FITC intensity histograms of the four samples. The vertical axis shows the intensity of annexin V-FITC and the horizontal axis depicts cell number. (C) Percentage of early apoptotic cells in the four groups. Results are presented as mean ± standard deviation of five samples in each treatment group. The differences between the groups were analyzed by ANOVA with Fisher's PLSD *post hoc* method. **P* < 0.05 versus control. FL1, annexin V-FITC; FL3, PI.

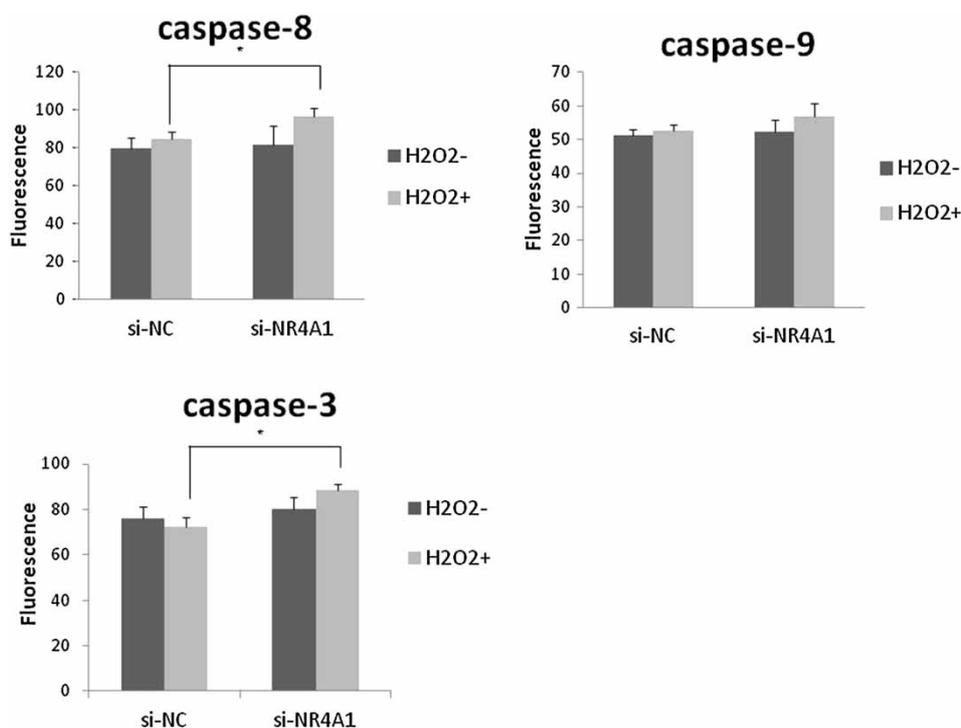


Figure 4 Assay for caspase-3, -8, and -9 activities. HUC-F2 cells transfected with si-NC or si-NR4A1 were treated without or with H₂O₂ at a concentration of 200 μ M for 2 hours. Caspase activity was measured using ApoAlert Caspase Assay plates. Each group contained four dishes. The caspase activity of each dish was measured in duplicate, and the average of the two measurements used. Results are presented as mean \pm standard deviation of four dishes in each treatment group. The differences between the groups were analyzed by ANOVA with Fisher's PLSD *post hoc* method. * $P < 0.05$ versus control.

GGUCCUGCACAGCUUGCUUGUCGA' was used (Fig. 2A). The other siRNA examined did not induce >80% inhibition. NR4A1 expression was reduced by 70.4, 87.2, and 83.7%, respectively. Next, HUC-F2 cells were transfected with si-NR4A1 for 48 hours, followed by treatment with 200 μ M H₂O₂ for 2–8 hours. NR4A1 mRNA was rapidly induced in HUC-F2 cells, and the most significant increase in expression was observed at the 2 hours time point (Fig. 2B). To establish whether or not NR4A1 mRNA expression is influenced by H₂O₂, gene expression profiling was performed with two house-keeping genes (β -actin and GAPDH), NR4A3 expression and additionally monitored to examine off-target effects. The gene expression patterns of NR4A3 were not significantly different. As shown in Fig. 1B, NR4A1 mRNA expression was four-fold higher upon treatment with 200 μ M H₂O₂ for 4 hours, compared with untreated cells. For cells treated si-NC under similar conditions, almost no induction was observed (Fig. 2B). This finding indicates that siRNA of negative control did not have any effect on NR4A1 expression. Si-NR4A1-transfected HUC-F2 cells were subsequently challenged with 0–300 μ M H₂O₂ for 24 hours. Growth of HUC-F2 cells transfected with si-NR4A1 was significantly reduced following exposure to 300 μ M H₂O₂ for 24 hours, as measured with the MTT assay (Fig. 2C).

Detection of apoptosis

To compare the proportion of early apoptotic cells, HUC-F2 cells were transfected with siRNA and treated with 200 μ M H₂O₂ at concentrations below that leading to reduced cell viability. Apoptosis was determined using FCM. HUC-F2 cells were transfected with si-NR4A1 for 48 hours, treated with 200 μ M H₂O₂ for 2 hours and measured with FCM (Fig. 3A4). Cells transfected with si-NC without H₂O₂ (Fig. 3A1), si-NR4A1 without H₂O₂ (Fig. 3A2), and si-NC with 200 μ M H₂O₂ (Fig. 3A3) were additionally analyzed using FCM (Fig. 3A). The peak of the annexin V-FITC intensity histogram for cells treated with si-NR4A1 and H₂O₂ was shifted rightward, compared with the control (Fig. 3B). The percentages of early apoptotic cells (bottom right) were additionally compared among the four samples. FCM analysis showed an increase in early apoptotic HUC-F2 cells transfected with si-NR4A1 and treated with H₂O₂ to 9.0% from 7.3% (si-NC with H₂O₂, $P = 0.013$) (Fig. 3C), suggesting a pro-survival or anti-apoptotic function of NR4A1.

Activation of caspase-3, -8, and -9

Caspase activation plays a central role in the execution of apoptosis. The key components of the biochemical pathways of caspase activation have been identified.²¹ Two pathways of caspase activation have been extensively characterized to date, specifically, cell surface

death receptor and the mitochondria-initiated pathways. In the cell surface death receptor pathway, activation of caspase-8 following its recruitment to the death-inducing signalling complex is the critical event that transmits the death signal. In the mitochondria-initiated pathway, caspase activation is triggered by the formation of a multimeric Apaf-1/cytochrome-c complex that is fully functional in recruiting and activating procaspase-9. Activated caspase-8 and -9 cleave and activate downstream caspases-3, -6, and -7. In our experiments, activities of caspase-3 and -8 of NR4A1 knockdown cells treated with 200 μM H_2O_2 were significantly higher than those of negative control cells. Caspase-9 activity was also elevated, but not to a significant extent (Fig. 4).

Discussion and conclusions

NR4A1 has been shown to induce apoptosis in a number of cell lineages exposed to pro-apoptotic stimuli by directly targeting mitochondria and promoting cytochrome-c release.^{12–15} Paradoxically, NR4A1 can act as a death inhibitory factor, blocking cell death induced by ceramide¹⁶ and TNF.¹⁷ In the current study, HUC-F2 cells treated with H_2O_2 at concentrations of 400 μM H_2O_2 or higher died, and growth stopped at 300 μM H_2O_2 . Expression of NR4A1 was induced by H_2O_2 in a concentration-dependent manner suggesting that NR4A1 plays a role in HUC-F2 cell death induction under oxidative stress conditions (Fig. 1).

NR4A1 silencing mediated via siRNA has led to a drastic reduction in cell growth/survival in many cancer cell lines.¹⁸ This reduced growth correlates well with increased levels of apoptosis. In this study, growth of si-NR4A1-transfected HUC-F2 cells treated with H_2O_2 was significantly reduced (Fig. 2). FCM analysis also showed an increased proportion of early apoptotic cells in HUC-F2 transfected with si-NR4A1 and treated with H_2O_2 (Fig. 3). The collective results clearly support a prosurvival or anti-apoptotic function of NR4A1.

Various apoptotic pathways exist that can be distinguished based on the adapters and initiator caspases involved. Most apoptotic programmes fall into either the extrinsic or intrinsic category. Extrinsic apoptosis is triggered by extracellular cues delivered in the form of ligands binding to death receptors (DR). The binding of a ligand to its DR recruits monomeric procaspase-8 protein to be recruited to the death-inducing signalling complex.²² Intrinsic apoptosis is also known as mitochondrial apoptosis since it depends on factors released from mitochondria. This pathway is activated by many cellular stress factors, including growth factor deprivation.²³ The initiator caspase responsible for the intrinsic apoptosis pathway is caspase-9, which is activated by dimerization

induced when the caspase-9 CARD domain binds to the adapter protein apoptotic protease-activating factor-1.²⁴ Activated caspase-8 and -9 cleave and activate downstream caspases, such as caspases-3, -6, and -7.²⁵ In this study, caspase-3 and -8 activities were elevated in cells transfected with si-NR4A1 and exposed to H_2O_2 , while that of caspase-9 activity was not significantly affected (Fig. 4). The data suggest that NR4A1 inhibits extrinsic apoptosis by reducing caspase-8 and caspase-3 activities.

NR4A1 has been previously implicated in cell growth/survival and apoptosis. In this study, we have demonstrated that NR4A1 inhibits apoptosis caused by oxidative stress in HUC-F2 cells. However, even upon NR4A1 knockdown, many of the cells survived. The most likely explanation for this finding is that NR4A2 and NR4A3 compensate for NR4A1 function, since all three orphan receptors bind the same responsive element of AAAGGTCA.¹⁸

Interestingly, NR4A1 expression varies in diverse cell types after stimulations with different reagents. NR4A1 translocation is reported to be involved in myocardial ischemia/reperfusion-induced apoptotic injury of cardiac myocytes subjected to metabolic syndrome.²⁶ It would be interesting to determine whether H_2O_2 treatment similarly causes cytoplasmic shuttling of NR4A1 in normal fibroblasts. Furthermore, to understand the physiological role of NR4A1 in normal fibroblasts, it is necessary to ascertain whether NR4A1-mediated inhibition of extrinsic apoptosis is specific to HUC-F2 cells or common in normal fibroblasts. Nevertheless, in the current study, we showed that NR4A1 acts as a regulator that inhibits extrinsic apoptosis in HUC-F2 cells during oxidative stress by reducing caspase-8 and -3 activities. Since NR4A1 plays an essential role in DNA repair,²⁷ we are further interested in determining whether inhibition of NR4A1 causes more DNA damage in normal fibroblasts which will be the focus of our future research.

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Disclaimer statements

Contributors Yuri Shimizu and Riko Miyakura conducted the experiments and Yuzuru Otsuka planned this research.

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Conflicts of interest None.

Ethics approval Ethics approval and consent was not needed.

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