The Orphan Nuclear Receptor NR4A1 (Nur77) Regulates Oxidative and Endoplasmic Reticulum Stress in Pancreatic Cancer Cells

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Abstract

NR4A1 (Nur77, TR3) is an orphan nuclear receptor that is overexpressed in pancreatic cancer and exhibits pro-oncogenic activity. RNA interference of NR4A1 expression in Panc-1 cells induced apoptosis and subsequent proteomic analysis revealed the induction of several markers of endoplasmic reticulum stress, including glucose-related protein 78 (GRP78), CCAAT/enhancer-binding protein-homologous protein (CHOP), and activating transcription factor-4 (ATF-4). Treatment of pancreatic cancer cells with the NR4A1 antagonist 1,1-bis(3-indolyl)-1-(p-hydroxyphenyl)methane (DIM-C-pPhOH) gave similar results. Moreover, both NR4A1 knockdown and DIM-C-pPhOH induced reactive oxygen species (ROS), and induction of ROS and endoplasmic reticulum stress by these agents was attenuated after cotreatment with antioxidants. Manipulation of NR4A1 expression coupled with gene expression profiling identified a number of ROS metabolism transcripts regulated by NR4A1. Knockdown of one of these transcripts, thioredoxin domain containing 5 (TXNDC5), recapitulated the elevated ROS and endoplasmic reticulum stress; thus, demonstrating that NR4A1 regulates levels of endoplasmic reticulum stress and ROS in pancreatic cancer cells to facilitate cell proliferation and survival. Finally, inactivation of NR4A1 by knockdown or DIM-C-pPhOH decreased TXNDC5, resulting in activation of the ROS/endoplasmic reticulum stress and proapoptotic pathways.

Implications: The NR4A1 receptor is pro-oncogenic, regulates the ROS/endoplasmic reticulum stress pathways, and inactivation of the receptor represents a novel pathway for inducing cell death in pancreatic cancer. Mol Cancer Res; 12(4); 527–38. ©2014 AACR.

Introduction

Nuclear receptor (NR) superfamily genes are critical regulators of homeostasis at all stages of development (1), and the 48 human nuclear receptors include the endocrine receptors, adopted orphan receptors, and orphan receptors. The NR4A orphan receptor subfamily consists of 3 orphan receptors (2, 3), namely NR4A1 (Nur77, TR3, NGFI-B), NR4A2 (Nurr1), and NR4A3 (Nor1), that were initially identified as immediate-early response genes induced by nerve growth factor in PC12 cells (4). The well-conserved DNA binding and C-terminal ligand binding domains of the NR4A receptors exhibit ~91% to 95% and ~60% homology, respectively, whereas their N-terminal A/B domains are highly divergent (5–7).

NR4A1 and NR4A2 bind as monomers and homodimers to NGFI-B response elements (NRBE) and Nur-responsive elements (NuRE), respectively. NR4A1 and NR4A2 (but not NR4A3) also form heterodimers with RXR and bind a DRE motif (8–10). NR4A receptors are induced by multiple stimuli/stressors and play essential roles in metabolic processes, inflammation, vascular function, steroidogenesis, and in the central nervous system. NR4A1 and NR4A2 are also overexpressed in multiple tumors and cancer cell lines (11, 12). RNA interference (RNAi) has been used to investigate the role of NR4A1 in cancer cells, and most studies indicate pro-oncogenic function where NR4A1 enhances both cancer cell proliferation and survival (11–20).

Zhang has summarized studies on a novel drug-dependent proapoptotic pathway in some cancer cell lines that is...
dependent on the nuclear export of NR4A1 and the subsequent formation of a mitochondria-associated NR4A1/bcl-2 proapoptotic complex (21). This has also led to development of peptide mimics that convert bcl-2 into an apoptotic complex, and paclitaxel, the taxane-derived anticancer drug, also exhibits comparable activity (22, 23). Multiple anticancer drugs and other apoptosis-inducers activate nuclear export of NR4A1, and there are reports that cytosporone B (CsnB) and related analogs may activate nuclear NR4A1 and also induce nuclear export of this receptor (24).

Knockdown of NR4A1 in lung and pancreatic cancer cell lines inhibits cell growth and induces apoptosis which is due, in part, to downregulation of the antiapoptotic gene survivin (19). Mechanistic studies showed that survivin expression is regulated by a p300/NR4A1/Sp1 complex bound to the proximal GC-rich sites of the survivin promoter (19). NR4A1 also binds and inactivates wild-type p53 (25), and NR4A1 silencing results in mTOR inhibition because of activation of p53 and p53-dependent induction of sestrin2, which in turn activates AMPK (20). 1,1-Bis(3'-indolyl)-1-(p-hydroxyphenyl) methane (DIM-C-pPhOH) has been characterized as an NR4A1 antagonist, which mimics the effects of NR4A1 silencing. DIM-C-pPhOH decreased cancer cell and tumor growth, activated p53-dependent inhibition of mTOR signaling, and downregulated survivin expression (19, 20).

NR4A1 silencing or treatment with DIM-C-pPhOH also altered pancreatic cancer cell morphology and fragmented the endoplasmic reticulum, which is typically observed in cells undergoing endoplasmic reticulum (ER) stress. Results of this study show that NR4A1 regulates reactive oxygen species (ROS) production and expression of genes such as thioredoxin domain containing 5 (TXNDC5) that maintain stress levels that are permissive for cancer cell growth and survival, and both chemical and RNAi-mediated inactivation of NR4A1 induce endoplasmic reticulum stress and death pathways in pancreatic cancer cells.

Materials and Methods

Cell lines and plasmids

Panc-1 and L3.6pl human pancreatic cancer cell lines were obtained and maintained as previously described (19). The TXNDC5 promoter (-1444/+25) reporter construct (pSESNN2-Luc) was purchased from Genecopoeia, Inc. All other reporter constructs have been previously described (19).

Antibodies, reagents, quantitative real-time PCR, Western blot analysis, and immunoprecipitation

NR4A1 antibody was purchased from Abcam, and TXNDC5, PGK1, and ATTP5A1 antibodies were purchased from GeneTex. XBP-1s (spliced XBP-1) and phosphorylated PERK (Ser713) antibodies were purchased from BioLegend. ATF4, CHOP, GRP78, β-actin, and IDH1 antibodies were purchased from Santa Cruz Biotechnology, and ATF6 antibody was purchased from Abgent. All remaining antibodies were purchased from Cell Signaling Technology. DIM-C-pPhOH was synthesized and purified in this laboratory as previously described (19). Reporter lysis buffer, luciferase reagent, and β-galactosidase (β-gal) reagent were supplied by Promega. Quantitative real-time PCR and Western blot analysis were undertaken as previously described (20). The real-time PCR primers for NR4A1 were purchased from Qiagen, and all other sequences of the primers used for real-time PCR were shown in Supplementary Table S1.

Two-dimensional gel electrophoresis and protein pathway analysis

Panc-1 cells were washed 3 times with ice-cold PBS. Cells were solubilized in a lysis buffer consisting of 5 mmol/L EDTA, 9.5 M urea, 4% (v/v) CHAPS, 65 mmol/L DTT, and protease inhibitors (Complete Kit; Roche Diagnostics). Samples (310 μL) containing 450 μg of proteins were loaded onto IPG strips (17 cm, pH 3-10 nonlinear, pH 5-8; Bio-Rad). The second dimension was performed in 12% SDS-polyacrylamide gels that were then stained with Coomassie Brilliant Blue G-250 and scanned at 300-dpi resolution. Protein spots were analyzed with ImageMaster Platinum software (GE Healthcare) according to the manufacturer’s procedures. Proteins altered over 1.5-fold were considered to be significantly changed (P > 0.01 by the Mann–Whitney test). The differentially expressed proteins were cut and digested, and dried sample were desalted with C18 Zip tips (Millipore) and were resuspended in 100 μL of an aqueous solution containing 5% acetic acid and 5% acetonitrile for liquid chromatography/tandem mass spectrometry (LC/MS-MS) analysis. Each sample solution was loaded onto a 0.1 to 150-mm Magic C18AQ reverse phase column (Michrom Bioreources, Inc.) in line after a nanotrap column using the Paradigm MS4 HPLC system (Michrom Bioreources, Inc.). Separation of the peptides was performed at 500 nL/min and was coupled to online analysis by tandem mass spectrometry using an LTQ ion trap mass spectrometer (ThermoElectron) equipped with a nanospray ion source (ThermoElectron). The peptides were detected in positive ion mode using a data-dependent method in which the 7 most abundant ions detected in an initial survey scan were selected for MS-MS analysis. The MS-MS spectra of two-dimensional (2D) samples were searched with Mascot Daemon (Matrix Science). The following MASCOT parameter settings were used: the peptide tolerance was 15 ppm and peptide charges and the MS-MS tolerance was 0.6 Da. One missed cleavages by trypsin were allowed, carbamidomethyl (C) was used as a fixed modification and oxidation (M) was used as variable modifications. When multiple proteins were identified in a single spot, the proteins with the highest number of peptides were considered as those corresponding to the spot, and the proteins with lower but significant scores were also recorded in the database.

Transmission electron microscopy

Cells were collected and fixed in 3% glutaraldehyde plus 2% paraformaldehyde in PBS for 2 hours, postfixed with 1% OsO4 for 2 hours, and stained for 1 hour in 1% aqueous uranyl acetate. The samples were then dehydrated with increasing concentrations of ethanol, infiltrated,
and embedded in Spurr low-viscosity medium. Ultrathin sections were cut using a Leica Ultracut microtome (Leica), counterstained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined under a JEM 1010 transmission electron microscope (JEOL USA Inc.).

Transfection, siRNA oligonucleotides, and reporter gene assay
Cells were transfected with 100 nmol/L of each siRNA duplex for 6 hours using LipofectAMINE 2000 reagent (Invitrogen), following the manufacturer’s protocol. The sequences of siNR4A1 oligonucleotides used were 5’-CAG UCC AGC CAU GCU CCU C d’TdT-3’. As a negative control, a nonspecific scrambled small inhibitory RNA (siScr) oligonucleotide was used (Qiagen) and all other siRNAs were purchased from Santa Cruz Biotechnology. Reporter gene assays were performed as previously described (20).

Measurement of intracellular level of ROS
OxiSelect Intracellular ROS Assay Kit (Cell Biolabs, Inc.) was used according to the manufacturer’s instructions. The treated cells were lysed and the amount of intracellular ROS was estimated from dichlorodihydrofluorescein (DCF) production measured by flow cytometry or a fluorometric plate reader (FLUOstar OPTIMA; BMG Labtechnologies) using excitation and emission wavelengths of 480 nm/530 nm. DNA fragmentation assay
After incubation with siNR4A1 or DIM-C-pPhOH, cells were subjected to DNA fragmentation assay according to the manufacturer’s instructions (Cell Death Detection ELISA-PLUS; Roche). Briefly, cells were lysed, cleared by centrifugation, and transferred into streptavidin-coated plate. Immunoreagent containing biotin-labeled antihistone antibody and peroxidase-conjugated antinucleosomal DNA antibody were then added to each well, and the plate was incubated for 2 hours with gentle shaking. After incubation, 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate solution was added to each well and the plate was incubated on a plate shaker at 250 rpm for 5 minutes. The absorbance was measured at 405 and 490 nm as test and reference wavelengths, respectively.

Intracellular Ca2+ assay
Intracellular cytosolic Ca2+ was measured using the Fluo-4 NW Calcium Assay Kit (Molecular Probes). After incubation with siNR4A1 or DIM-C-pPhOH, cells were loaded with Fluo-4 according to the manufacturer’s instructions. The fluorescence of Fluo-4 was measured by a fluorometric plate reader (FLUOstar OPTIMA; BMG Labtechnologies) using excitation at 485 nm and emission at 520 nm.

Protein pathway analysis
After protein identification, the accession numbers and fold changes of the differentially expressed proteins were tabulated in Microsoft Excel and imported into IPA (Ingenuity System). IPA is a software application that enables to identify the biologic mechanisms, pathways, and functions matching a particular dataset of proteins. IPA is based on a database obtained by abstracting and interconnecting a large fraction of the biomedical literature according to an algorithm integrating protein functions, cellular localization, small molecules, and disease inter-relationships. The networks are displayed graphically as nodes, representing individual proteins and edges representing the biologic relation between nodes. Canonical pathway analysis within IPA utilizes well-characterized metabolic and cell signaling pathways, which are generated before data input and on which identified proteins are overlaid.

Isolation of RNA and microarray analysis
Total RNA was extracted from Panc-1 cells by using a mirVanaTM miRNA Isolation Labeling Kit (Ambion Inc.). The total RNA was quantified by using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technology). The total RNA samples with adequate RNA quality index (>7) were used for microarray analysis. Sample labeling was performed with RNA Amplification Kit according to the manufacturer’s instructions (Applied Biosystems). We used 700 ng of total RNA for labeling and hybridization on HumanHT-12 v4 expression beadchip (Illumina Inc.). After the bead chips were scanned with a BeadArray Reader (Illumina), the microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data (LIMMA) package in the R language (http://www.r-project.org). BRB-ArrayTools were primarily used for statistical analysis of gene expression data and the t test was applied to identify the genes significantly different between 2 groups when compared. Cluster analysis was performed using the software programs Cluster and Heatmap generated by Treeview.

DNA-binding assay and chromatin immunoprecipitation assay
GC-rich DNA binding of NR4A1 was measured using a Universal EZ-TFA Transcription Factor Assay Chemiluminescent Kit (Upstate Biotechnology, Inc.) according to the manufacturer’s protocol. A biotinylated double-stranded oligonucleotide containing the putative NBRE sequence of TXNDC5 was used as a capture probe and an unlabeled oligonucleotide containing the identical consensus sequence as the capture probe was used as a competitor. A negative control without the capture probe was also used in each assay. The chromatin immunoprecipitation (ChiP) assay was performed using Chip-IT Express Magnetic Chromatin Immunoprecipitation Kit (Active Motif) as previously described (20). The TXNDC5 primers that contain the NBRE were 5’-CTA ATT CAG GTG CAA ACC CCA CC-3’ (sense) and 5’-AGC GGC GGT GAA CAC ACG AAG TA-3’ (antisense).

Statistical analysis
Statistical significance of differences between groups was analyzed using the Student t test. The results are expressed as means with error bars representing 95% confidence intervals.
for 3 experiments for each group unless otherwise indicated, and a P value of less than 0.05 was considered statistically significant. All statistical tests were 2-sided.

Results
Inactivation of NR4A1 induces endoplasmic reticulum stress and apoptosis
Silencing NR4A1 or inactivation of the receptor by DIM-C-pPhOH inhibited pancreatic cancer cell and tumor growth through decreased expression of some NR4A1-regulated prosurvival genes such as survivin (19). In this study, we have used a proteomic and gene array approach to identify other key genes regulated by this receptor. Silencing of NR4A1 by RNA interference (RNAi, siNR4A1) followed by 2D gel electrophoresis (Fig. 1A) and mass spectrometry analysis of individual spots, identified 38 proteins induced or suppressed by >2-fold (Supplementary Table S2). The functional distribution (Fig. 1B) and canonical pathways (Fig. 1C) resulting from NR4A1 knockdown demonstrate that this receptor plays a key role in regulating endoplasmic reticulum stress in Panc-1 cells and this represents a hitherto unknown function for this receptor. Figure 1D illustrates that after knockdown of NR4A1 in Panc-1 cells, there is increased intensity of gel spots identified by mass spectrometry as GRP78 and ERP60, 2 endoplasmic reticulum chaperone proteins, and decreased expression of the mitochondrial ATP5A1 and cytosolic PGK1 proteins. In a separate experiment, Western blot analysis of whole cell lysates from Panc-1 cells transfected with siNR4A1 or siScr (nonspecific oligonucleotide) confirmed induction of the endoplasmic reticulum stress markers (GRP78 and ERP60) and down-regulation of ATP5A1 and PGK1 (Fig. 1D).

The novel observation that NR4A1 regulates stress in pancreatic cancer cells was further confirmed in Panc-1 cells transfected with siNR4A1 and examined by transmission electron microscopy (Fig. 2A). The results show that typical structural features of the endoplasmic reticulum are disrupted and fragmented. Western blot analysis of Panc-1 cell lysates after NR4A1 silencing confirmed the induction of several endoplasmic reticulum stress markers, including ATF4, XBP-1s, GRP78, and CHOP (Fig. 2B). Moreover, NR4A1 silencing also resulted in induction of apoptosis as evidenced by induction of cleaved caspase-8, caspase-3, and caspase-7 and PARP and decreased expression of Bid (p20; Fig. 2B), and these responses are typically observed after induction of endoplasmic reticulum stress (26–28). NR4A1 silencing in L3.6pL pancreatic cancer cells also induced endoplasmic reticulum stress (p-PERK, ATF4, XBP-1s, and CHOP) and apoptosis (cleaved caspase-8 and PARP; Fig. 2C), and NR4A1 silencing in MCF-7 breast and RKO colon cancer cells (Fig. 2D; also MDA-MB-231 and Jurkat cells, data not shown) confirmed that this receptor also regulated endoplasmic reticulum stress in several cancer cell lines. Moreover, tumor lysates from mice treated with corn oil (control) or DIM-C-pPhOH (30 mg/kg/d; ref. 19) also showed enhanced expression of endoplasmic reticulum stress markers in the latter group (Supplementary Fig. S1A).

Because CHOP is a key stress-induced proapoptotic protein, we further investigated the role of CHOP in mediating the effects of NR4A1 silencing in Panc-1 cells (Fig. 3A). Knockdown of NR4A1 enhanced activation of caspase-8 and caspase-7 (cleaved) and PARP cleavage and, in cells transfected with siNR4A1, the proapoptotic responses were attenuated in cells cotransfected with siCHOP. These results confirm a role for CHOP and endoplasmic reticulum stress in mediating siNR4A1-induced apoptosis. We also observed that both siNR4A1 and DIM-C-pPhOH induce LC3-II, a marker of autophagy and siCHOP attenuated this response (Supplementary Fig. S1B and S1C), indicating that CHOP plays a role in endoplasmic reticulum stress–induced autophagy as previously reported (29).

Inactivation of NR4A1 induces ROS
ROS is an important inducer of endoplasmic reticulum stress, and therefore we examined the effects of NR4A1 silencing on induction of ROS using the cell permeant dichlorofluorescein diacetate (DFCH-DA) probe and observed that cells transfected with siNR4A1 exhibited significantly induced ROS, which was inhibited after cotreatment with glutathione (GSH; Fig. 3B). Moreover, in Panc-1 cells transfected with siScr or siNR4A1, the induction of endoplasmic reticulum stress (p-PERK, XBP-1s, and CHOP) and apoptosis (cleaved caspase-8 and caspase-7 and PARP) markers after NR4A1 silencing were partially reversed after cotreatment with GSH (Fig. 3C). The role of NR4A1 silencing in inducing ROS-dependent endoplasmic reticulum stress was also confirmed in L3.6pL pancreatic cancer cells in which transfection with siNR4A1 increased markers of endoplasmic reticulum stress (p-PERK, XBP-1s, ATF4, and CHOP) and apoptosis (cleaved caspase-8 and PARP) and these responses were attenuated by cotreatment with GSH (Fig. 3D).

Previous studies have shown that DIM-C-PhOH acts as an NR4A1 antagonist and mimics the effects of NR4A1 silencing (19, 20), and Fig. 4A shows DIM-C-PhOH significantly increased expression of markers of endoplasmic reticulum stress (ATF4, XBP-1s, and CHOP) and apoptosis (decreased Bid and increased cleavage of caspase-8 and caspase-7 and PARP; Fig. 4A), which was comparable to the effects of siNR4A1 (Fig. 2B). Moreover, using a quantitative DNA fragmentation assay (Supplementary Fig. S2A), we showed that both siNR4A1 and DIM-C-PhOH significantly induced DNA damage, and both treatments also increased endoplasmic reticulum calcium flux (Supplementary Fig. S2B) associated with endoplasmic reticulum stress. DIM-C-pPhOH-induced ATF4, XBP-1s, CHOP, cleaved caspase-8, and PARP was attenuated in Panc-1 cells infected with adenovirus overexpressing NR4A1 (Fig. 4B). Moreover, like NR4A1 silencing, inactivation of this receptor by DIM-C-pPhOH also induced ROS, which was attenuated after cotreatment with GSH (Fig. 4C), and DIM-C-pPhOH-induced markers of endoplasmic reticulum stress (CHOP, ATF4, XBP-1s, and CHOP) and apoptosis (cleaved caspase-8 and caspase-7 and PARP) were also decreased after cotreatment with GSH (Fig. 4D).
Figure 1. 2D-PAGE gels showing differentially expressed proteins in Panc-1 cells transfected with siNR4A1 and functional classification of the proteins. Cells were transfected with either siScr or siNR4A1 for 60 hours, and whole cell lysates (400 μg of protein) were prepared, and the pH gradient in the first dimension was from 3 to 10. The second dimension was a 12% acrylamide SDS-PAGE. Gels were stained by colloidal staining with Coomassie blue G250 (A). Arrows point to the proteins of interest, and the numbers assigned to the spots correspond to the numbers listed in Supplementary Table S2. Functional distribution (B) and canonical pathway (C) of the 38 identified proteins. Assignments were made based on information from the NCBI, the Swiss-Prot/TrEMBL Protein Knowledge Base, and the Ingenuity Pathways Knowledge Base. Enlarged images of protein spots for which image analysis software reported ≥2-fold differences in accumulation in siNR4A1-transfected cells are shown (D, left). Panc-1 cells were transfected with either siScr or siNR4A1 for 60 hours, and whole cell lysates were analyzed by Western blot analysis (D, right); ER, endoplasmic reticulum.
Inactivation of NR4A1 induces genes involved in ROS metabolism

Thus, siNR4A1 or inactivation of NR4A1 by DIM-C-pPhOH induced ROS-dependent endoplasmic reticulum stress, and identification of NR4A1-regulated genes involved in ROS metabolism was further investigated by RNAi using Illumina beadchip arrays (Fig. 5A and Supplementary Table S3) and several canonical pathways were also affected (Supplementary Fig. S3). Only 3 potential NR4A1-regulated genes involved in ROS metabolism were decreased in Panc-1 cells, namely, IDH1, GLRX, and TXNDC5, and in a separate experiment, we confirmed by real-time PCR that these genes are regulated by NR4A1 (Fig. 5A). The potential roles of the NR4A1-regulated IDH1, GLRX, and TXNDC5 in mediating endoplasmic reticulum stress were investigated by RNAi (Fig. 5B). GLRX protein expression was minimal and endoplasmic reticulum stress genes were not induced by siGLRX; however, knockdown of IDH1 induced CHOP and cleaved caspase-8 and PARP and this was accompanied by induction of ROS (Fig. 5C). We also observed that silencing of TXNDC5 by RNAi (siTXNDC5) resulted in the induction of ROS, which was attenuated after cotreatment with GSH; transfection of Panc-1 cells with siTXNDC5 also induced expression of ATF4, CHOP, cleaved caspase-8, and PARP and these responses were attenuated in cells transfected with siScr or siNR4A1 for 72 hours, and whole cell lysates were analyzed by Western blot analysis. β-Actin was used as a loading control.

![Figure 2](https://example.com/fig2.png)

Figure 2. Knockdown of NR4A1 induces endoplasmic reticulum stress and apoptosis in multiple cancer cell lines. A, Panc-1 cells were transfected with either siScr or siNR4A1, and TEM analysis was performed at 60 hours after transfection. The structure of the endoplasmic reticulum (ER; arrows) in cells transfected with siNR4A1 showed morphologic disorders. B–D, Panc-1, L3.6pl-, MCF-7/RKO cells, respectively, were transfected with either siScr or siNR4A1 for 72 hours, and whole cell lysates were analyzed by Western blot analysis. β-Actin was used as a loading control.
has a putative NBRE at −348 and, in Panc-1 cells transfected with each indicated siRNA for 72 hours, and whole cell lysates were analyzed by Western blot analysis. B-D, cells were transfected with siScr or siNR4A1 for 6 hours. At 60 hours after transfection, ROS production was measured by the oxidation of nonfluorescent DCFH-DA to fluorescent DCF using a fluorescence plate reader (B). \(^{*}P < 0.001\) vs. siScr without reduced GSH. At 24 hours after transfection, the cells were treated with GSH for an additional 48 hours and whole cell lysates were analyzed by Western blot analysis (C and D). β-Actin was used as a loading control. The multiple lanes represent lysates from different experiments.

NR4A1 Regulates Stress in Pancreatic Cancer Cells

Discussion

NR4A1 is a unique nuclear receptor that is overexpressed in cancer cells and tumors, and results of RNAi demonstrate the pro-oncogenic activity of this receptor (12). Some cancer cell lines treated with phorbol esters, cyclic retinoids, and other apoptosis-inducing agents induce nuclear export of NR4A1, which forms a proapoptotic complex with bcl-2
This unusual NR4A1-dependent drug-induced apoptotic pathway does not require the DNA binding domain of NR4A1 and is blocked by nuclear export inhibitors such as leptomycin B (LMB). Studies in this laboratory have investigated the effects of 1,1-bis(3'-indolyl)-1-(p-substituted phenyl)methane (C-DIM) derivatives on NR4A1-mediated transactivation and DIM-C-pPhOH was identified as an NR4A1 antagonist that inhibited nuclear NR4A1-mediated responses and mimicked the effects of NR4A1 knockdown by RNAi (19, 20). DIM-C-pPhOH and siNR4A1 inhibited pancreatic and lung cancer cell proliferation, and DIM-C-pPhOH also inhibited tumor cell growth. Mechanistic studies showed that NR4A1 regulated expression of prosurvival and growth-promoting genes through p300–NR4A1–Sp1 complex interactions with GC-rich gene promoter sequences (e.g., survivin, ref. 19), and NR4A1 also activated mTOR (20) by inhibiting the function of p53 (25). Knockdown of NR4A1 by RNAi or inactivation of the receptor by DIM-C-pPhOH reversed these responses, resulting in inhibition of cell and tumor growth, induction of apoptosis and inhibition of mTOR (19, 20). However, microscopic examination of Panc-1 cells transfected with siNR4A1 or treated with DIM-C-pPhOH showed that NR4A1 was important for regulating stress levels because loss of this receptor resulted in significant loss and fragmentation of endoplasmic reticulum structure (Fig. 2A), indicative of endoplasmic reticulum stress.

We initially used a proteomics approach to identify stress-related gene products that are modulated after silencing NR4A1 (Fig. 1B and C), and both siNR4A1 and DIM-C-pPhOH induced several prototypical endoplasmic reticulum stress-activated genes that are involved in stress responses, including ATF4, XBP-1s, CHOP, c-C8, c-C7, c-PARP, and β-Actin. Whole cell lysates were analyzed by Western blot analysis (NR4A1 protein levels were induced 4-fold by Ad-NR4A1), and cells were treated with DMSO or DIM-C-pPhOH for 18 hours, and ROS production was measured by the oxidation of nonfluorescent DCFH-DA to fluorescent DCF using either flow cytometric analysis (left) or a fluorescence plate reader (right). D, cells were treated with DMSO or DIM-C-pPhOH in the presence or absence of GSH for 24 hours and whole cell lysates were analyzed by Western blot analysis. β-Actin was used as a loading control.

Figure 4. DIM-C-pPhOH, the NR4A1 inactivator, induces endoplasmic reticulum stress–mediated apoptosis by increasing ROS production in Panc-1 cells. A, cells were treated with either dimethyl sulfoxide (DMSO) or DIM-C-pPhOH for 24 hours. B, cells were transfected with either Ad-Null or Ad-NR4A1 for 12 hours, and treated with DIM-C-pPhOH (25 μmol/L) for another 24 hours. Whole cell lysates were analyzed by Western blot analysis (NR4A1 protein levels were induced 4-fold by Ad-NR4A1). C, cells were treated with DMSO or DIM-C-pPhOH for 18 hours, and ROS production was measured by the oxidation of nonfluorescent DCFH-DA to fluorescent DCF using either flow cytometric analysis (left) or a fluorescence plate reader (right). D, cells were treated with DMSO or DIM-C-pPhOH in the presence or absence of GSH for 24 hours and whole cell lysates were analyzed by Western blot analysis. β-Actin was used as a loading control.
stress–related genes. Analysis of the induced and repressed proteins by mass spectrometry demonstrated that NR4A1 silencing in Panc-1 cells resulted in induction of the stress proteins GRP78 and ERp60 and several other classes of proteins, including the mitochondrial and cytosolic ATP5A and PGK1 proteins, respectively (Fig. 1D). NR4A1 silencing or treatment with DIM-C-pPhOH induced several other endoplasmic reticulum stress–related genes, including ATF4, XBP-1s, and CHOP, as well as several markers of apoptosis that are associated with activation of endoplasmic reticulum stress (refs. 26–28 and Figs. 2B and 4A). Moreover, many of the same markers of endoplasmic reticulum stress and apoptosis were also induced in L3.6pL pancreatic, MCF-7 breast, and RKO colon cancer cells transfected with siNR4A1 (Fig. 2C and D), suggesting that NR4A1 serves as an important regulator (inhibitor) of endoplasmic reticulum stress in cancer cells.

Based on results of CHOP silencing (Fig. 3A), it was also evident that this gene product plays a critical role in siNR4A1 and DIM-C-pPhOH-induced endoplasmic reticulum stress and subsequent induction of cleaved caspases and PARP. Studies with several anticancer agents have demonstrated a direct linkage between drug-induced ROS and ROS-dependent induction of CHOP and downstream responses, including apoptosis (30–32). For example, the polyherbal formulation zyflamend induces ROS and endoplasmic reticulum stress (including CHOP) in colon and pancreatic cancer cells (33) and, using HCT116 colon...
cancer cells as a model, it was shown that cotreatment with antioxidants reverse zyflamend-induced CHOP and apoptosis. The observations are consistent with our data showing that both siNR4A1 and DIM-C-pPhOH induced ROS in Panc-1 cells (Figs. 3B and 4C) and the antioxidant GSH significantly inhibited induction of CHOP and other markers of endoplasmic reticulum stress and apoptosis (Figs. 3D and 4D). These results suggest that NR4A1 attenuates stress levels in pancreatic cancer cells by regulating ROS metabolism and production. GSH only partially protected against NR4A1-dependent stress induced by DIM-C-pPhOH because C-DIM compounds induce stress via direct perturbation of mitochondria (34, 35).

Results of gene array studies showed that only 3 ROS metabolism genes were suppressed in Panc-1 cells transfected with siNR4A1 and these include IDH1, GLRX, and TXNDC5 (Fig. 5A). IDH1 plays a critical role in the citric acid cycle and the generation of reducing equivalents, and the relative expression of wild-type and mutant IDH1 have both functional and prognostic significance in cancer (36, 37).

Glutaredoxin (GLRX) is a thioredoxin that contributes to the cellular redox status and some GLRX genes are overexpressed in human lung and breast tumors (38, 39). TXNDC5 is a member of the thioredoxin family of endoplasmic reticulum proteins that contain a disulfide isomerase-like (PDI) domain (40–42). Transfection of Panc-1 cells with siNR4A1 decreases expression of all 3 genes; however, GLRX protein levels are low and knockdown of GLRX by RNAi does not activate endoplasmic reticulum stress or apoptosis, whereas silencing IDH1 and TXNDC5 induces endoplasmic reticulum stress and apoptosis (Fig. 5C and D). Induction of endoplasmic reticulum stress and apoptosis markers were more pronounced in Panc-1 cells transfected with siTXNDC5 versus siIDH1 (Fig. 5C and D), and results of ChIP assays...
and transfection with the TXNDC5 promoter construct confirmed that TXNDC5 is directly regulated by NR4A1. The parallel results observed after knockdown of NR4A1 or TXNDC5 or after treatment with the NR4A1 antagonist (DIM-C-pPhOH) suggest that regulation of TXNDC5 expression by NR4A1 is an important pro-oncogenic function of this receptor, which serves to maintain sufficiently low stress levels that facilitate cancer cell growth and survival. TXNDC5 expression is upregulated in non–small cell lung cancer, colorectal adenomas, and tumors of the cervix, uterus, and stomach (compared with nontumor tissue; refs. 41, 43–45), and overexpression of TXNDC5 in gastric cancer cells increased proliferation and migration and decrease apoptosis (46). Although data mining of patient-derived pancreatic cancer arrays did not detect higher TXNDC5 mRNA expression in tumor versus nontumor tissue (data not shown), results of this study demonstrate that TXNDC5 is an important NR4A1-regulated gene that significantly contributes to the pancreatic cancer cell phenotype. Thus, NR4A1 inactivates p53 to activate mTOR signaling (20), regulates expression of genes with GC-rich promoters (e.g., survivin and bcl-2; ref. 19), and regulates expression of genes such as TXNDC5 to maintain low levels of ROS-induced stress in cancer cells. These NR4A1-regulated genes/pathways highlight the pro-oncogenic activity of this receptor and the importance of this gene as a target for DIM-C-pPhOH and related compounds that are currently being developed as NR4A1 antagonists for cancer chemotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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## The Orphan Nuclear Receptor NR4A1 (Nur77) Regulates Oxidative and Endoplasmic Reticulum Stress in Pancreatic Cancer Cells

Syng-Ook Lee, Un-Ho Jin, Jeong Han Kang, et al.


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