IGF-1 induces expression of zinc-finger protein 143 in colon cancer cells through phosphatidylinositide 3-kinase and reactive oxygen species

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Abbreviations: DPI, diphenyleneiodonium; GIPC, GAIP interacting protein, C-terminus; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; MAPK, mitogen-activated protein kinase; MDC, monodansylcadavarine; NAC, N-acetyl Cysteine; PI3-kinase, phosphatidylinositide 3-kinase; ROS, reactive oxygen species; ZNF143, zinc-finger protein 143

Abstract

Expression of zinc-finger protein 143 (ZNF143), a human homolog of the *Xenopus* transcriptional activator protein Staf, is induced by various DNA-damaging agents including etoposide, doxorubicin, and γ-irradiation. ZNF143 binds to cisplatin-modified DNA, and its levels are increased in cancer cells that are resistant to anticancer drugs, including cisplatin, suggesting that it plays a role in carcinogenesis and cancer cell survival. However, the mechanism of ZNF143 induction in cancer cells remains unclear. Both insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) have been reported to be overexpressed in cancer cells and to be related to anticancer drug resistance, but the identity of the relevant signaling mediators is still being investigated. In the present study, we observed that IGF-1 was able to induce ZNF143 expression in HCT116 human colon cancer cells and that wortmannin, an inhibitor of phosphatidylinositide 3-kinase (PI3-kinase), inhibited this induction, as did diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, and monodansylcardavarine (MDC), a receptor internalization inhibitor. Treatment with MDC decreased the IGF-1-stimulated generation of reactive oxygen species. Taken together, these data suggest that IGF-1 induces ZNF143 expression in cancer cells via PI3-kinase and reactive oxygen species generation during receptor internalization.

Keywords: 1-phosphatidylinositol 3-kinase; drug resistance, neoplasm; insulin-like growth factor I; reactive oxygen species; ZNF143 protein, human

Introduction

Insulin-like growth factor-1 (IGF-1) is thought to regulate a variety of cellular processes, including cell survival, by binding to the IGF-1 receptor (IGF-1R) on cell surfaces. Ligand binding stimulates IGF-1R to initiate a cascade of intracellular tyrosine phosphorylation events; these events recruit insulin receptor substrate (IRS) proteins, Shc, and collagen (among other proteins) to the receptor, resulting in the activation of transcription factors involved in cell survival and proliferation (LeRoith and Roberts, 2003; Ouban *et al*., 2003; Miller and Yee, 2005; Tao *et al*., 2007; Pollak, 2008; Rodon *et al*., 2008).

Some research findings have implicated IGF-1/IGF-1R signaling in drug resistance and DNA repair mechanisms. For example, in both mouse embryo fibroblasts and breast tumor cells, enhanced radioresistance is proportional to the IGF-1R protein level (Turner *et al*., 1997), and in keratinocytes, delayed apoptosis in response to ultraviolet B occurs via IGF-1-mediated activation of the serine/threonine kinase Akt, resulting in enhanced repair of DNA cyclobutane thymidine dimers (Decraene *et al*., 2002). In addition, hyperactivation of the IGF-1R signaling pathway is an essential event in the development of cisplatin resistance by ovarian cancer cells (Eckstein *et al*., 2009). Trojanek and colleagues have shown that IGF-1 protects against cisplatin-induced cytotoxicity and that IGF-1/IRS1 participates in homologous recombination-directed DNA repair by regulating Rad51 localization, thereby supporting genome stability (Trojanek *et al*., 2003). However, the identities of the factors induced by IGF-1/IGF-1R signaling and the mechanisms by which

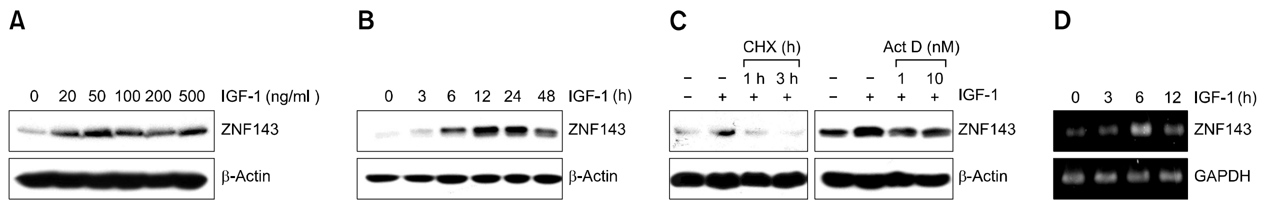

Figure 1. IGF-1 treatment induces ZNF143 expression in HCT116 cells. (A) Cells were plated on a 60-mm dish, grown for 24 h in DMEM containing 10% FBS, serum-starved for 24 h, incubated with the indicated concentrations of IGF-1 for 6 h, harvested, and analyzed for ZNF143 expression by immunoblotting. (B, D) Cells were grown and serum-starved as in (A), incubated with IGF-1 (100 ng/ml) for the indicated lengths of time, lysed, and analyzed for ZNF143 expression by immunoblotting (B) or RT-PCR (D). (C) Cells were grown and serum-starved, incubated with IGF-1 (100 ng/ml) for 6 h in the presence of cycloheximide (CHX; 35 μM), actinomycin D (ActD; 1 or 10 nM), or DMSO, lysed, and analyzed for ZNF143 expression by immunoblotting. Cycloheximide and actinomycin D were added 1-3 h and 16 h, respectively, prior to IGF-1 treatment. Results shown are representative of at least three independent experiments.

they enhance cell survival are unknown.

Zinc-finger protein 143 (ZNF143) is a human homolog of xenopus transcriptional activator staf and binding sites for ZNF143 have been found in approximately 2,000 mammalian promoters (Myslinski *et al*., 2006). ZNF143 binds to cisplatin-modified DNA and its expression can be induced by DNA-damaging agents, including etoposide, doxorubicin, and γ-irradiation (Ishiguchi *et al*., 2004). ZNF143 expression levels are reportedly increased in cancer cells that are resistant to anticancer drugs such as cisplatin, suggesting that the protein plays a role in carcinogenesis and cancer cell survival (Wakasugi *et al*., 2007). However, the mechanisms by which ZNF143 is induced and enhances cancer cells survival remain unclear.

Here, we investigated the possibility that IGF-1 treatment would induce ZNF143 expression in cancer cells and sought to identify the possible mediators of this induction. We also used ZNF143 expression knockdown experiments to examine whether ZNF143 might be important in cancer cell resistance to cisplatin-induced cell death.

Results and Discussion

IGF-1 induces ZNF143 protein expression in HCT116 cells

Although the induction of ZNF143 has been implicated in drug resistance, it remains to be elucidated how ZNF143 is induced in cancer cells and whether endogenous growth factors or cytokines in cancer cells might induce ZNF143 expression to enhance cell survival. Interestingly, IGF-1 was shown to protect against cisplatin-induced cytotoxicity and to be involved in homologous recombination-directed DNA repair by regulating Rad51 localization (Trojanek *et al*., 2003), which made us hypothesize that IGF-1 may be one of growth factors to regulate ZNF143 expression in cancer cells to promote cancer cell survival. To verify this hypothesis, first we investigated whether IGF-1 induces ZNF143 expression in HCT116 cells. Toward this end, HCT116 cells were serum-starved for 24 h and then incubated with various concentrations (0-500 ng/ml) of IGF-1 for various lengths of time (0-48 h). IGF-1 induction of ZNF143 became apparent after 6-h incubation with 20 ng/ml IGF-1 and increased as the IGF-1 concentration increased up to 100 ng/ml (Figure 1A and Supplemental Data Figure S2). At 100 ng/ml IGF-1, ZNF143 induction became apparent at 3 h and increased up to 12 h (Figure 1B). We next investigated whether IGF-1 affects ZNF143 expression through a transcriptional or post-transcriptional mechanism. HCT116 cells were incubated with 100 ng/ml IGF-1 for 6 h in the presence or absence of inhibitors of transcription (actinomycin D; 1 or 10 nM) or translation (cycloheximide; 35 μM), and ZNF143 expression was analyzed by immunoblotting (Figure 1C). Actinomycin D was added 16 h prior to IGF-1 addition, and cycloheximide was added 1 or 3 h prior to IGF-1 addition. As shown in Figure 1C, IGF-1-stimulated induction of ZNF143 expression was strongly inhibited by actinomycin D. In addition, ZNF143 induction by IGF-1 was shown by RT-PCR (Figure 1D), suggesting that the induction mechanism of ZNF143 by IGF-1 involves transcriptional regulation.

Phosphatidylinositol-3-kinase (PI3-kinase) and NADPH oxidase activities contribute to IGF-1-induced ZNF143 expression in HCT116 cells

IGF-1 activates various signaling pathways that enhance cell survival and proliferation, including pathways mediated by PI3-kinase/Akt (Xu *et al*., 1999; Kenchappa *et al*., 2004) and mitogen-activated protein kinases (MAPKs) (Chow *et al*.,

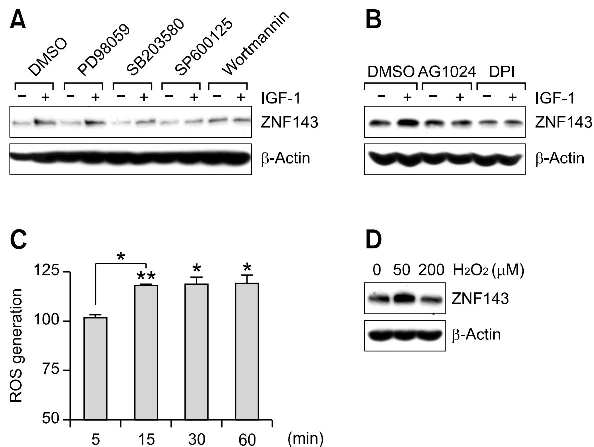

Figure 2. Inhibition of PI3-kinase, NADPH oxidase, or IGF-1R attenuates IGF-1-induced ZNF143 expression in HCT116 cells. (A, B) Cells were treated with various inhibitors or DMSO (vehicle). IGF-1 (100 ng/ml) was added 30 min later, and the cells were incubated for 6 h before lysis, SDS-PAGE, and immunoblotting to detect ZNF143 expression. In (A), the inhibitors were PD98059 (10 μM), SB203580 (10 μM), 10 μM SP600125 (10 μM), and wortmannin (100 nM). In (B), the inhibitors were AG1024 (5 μM) and DPI (10 μM). Results shown are representative of at least three independent experiments. (C) Cells were treated with IGF-1 (100 ng/ml) for 5-60 min as indicated. H_2DCFDA was added 10 min before harvest. Harvested cells were analyzed for DCF fluorescence reflecting relative ROS levels (arbitrary units) on a FACS Calibur instrument. Results shown are representative of at least three independent experiments. Data are expressed as means ± standard error (S.E.) of at least three independent experiments. Statistical significance was assessed using paired Student's t-tests (*, $P < 0.05$; **, $P < 0.002$). (D) Cells were incubated with H_2O_2 (0, 50, or 200 μM) for 6 h before lysis, SDS-PAGE, and immunoblotting to detect ZNF143 expression. Results shown are representative of at least three independent experiments.

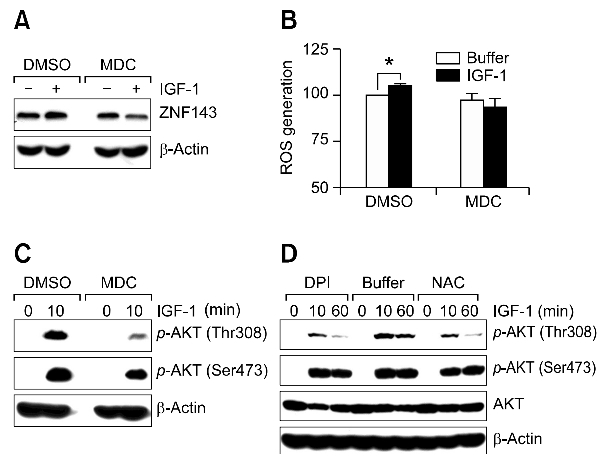

Figure 3. MDC, a receptor internalization inhibitor, inhibits IGF-stimulated ZNF143 induction through ROS and Akt in HCT116 cells. (A-C) Cells were incubated with MDC (50 μM) or DMSO (vehicle) for 30 min, and treated with IGF-1 for 6 h (A), 30 min (B), or 10 min (C). They were harvested and analyzed for ZNF143 expression using immunoblotting (A, C) and for ROS generation using flow cytometry, as described in Figure 2C (B). Results shown are representative of at least three independent experiments. Data are expressed as means ± S.E. of at least three independent experiments. Statistical significance was assessed using paired Student's *t*-tests (*, $P < 0.05$). (D) Serum- starved cells were exposed to IGF-1 for 0, 10, or 60 min in the presence of DPI (10 μM), NAC (1 mM), or DMSO, and Akt phosphorylation at residues Thr^{308} and Ser^{473} was analyzed by immunoblotting. Results shown are representative of at least three independent experiments.

1998; Girnita *et al*., 2007). IGF-1 also increases reactive oxygen species (ROS) generation in treated cells and contributes to the proliferation with the help of GAIP interacting protein, C-terminus (GIPC) (Choi *et al*., 2010) and migration of vascular smooth muscle cells *via* Nox4 and Rac1 (Meng *et al*., 2008; Vardatsikos *et al*., 2009). To verify which signaling pathways are involved in IGF-1-induced ZNF143 expression, HCT116 cells were incubated with IGF-1 in the presence of PD98059 (a MAPK kinase inhibitor), SB203580 (a p38 kinase inhibitor), SP600125 (a c-Jun amino-terminal kinase inhibitor), wortmannin (a PI3-kinase inhibitor), or DMSO (vehicle) (Figure 2A and Supplemental Data Figure S1). While the effect of SB203580 and SP600125 on IGF-1-induced ZNF143 expression was subtle, wortmannin strongly attenuated this induction, suggesting that PI3-kinase plays an important role in the IGF-1 signaling pathway leading to ZNF143 expression. IGF-1 induction of ZNF143 was also inhibited by AG1024, a receptor tyrosine kinase inhibitor specific for IGF-1R, and by diphenyleneiodonium sulfate (DPI), an NADPH oxidase inhibitor (Figure 2B), suggesting that the tyrosine kinase activity of IGF-1R and the ROS generated by the NADPH oxidase-dependent cascade mediate the IGF-1 signal in HCT116 cells. When we measured ROS generation in IGF-1-stimulated HCT116 cells exposed to IGF-1 for various lengths of time using flow cytometric analysis of DCF fluorescence, we found that ROS levels rose within 5 min of initiation of IGF-1 treatment, were maximal after 15 min of treatment, and remained high for at least 1 h (Figure 2C). To confirm the role of ROS in ZNF143 induction by IGF-1, starved HCT116 cells were incubated with H_2O_2 and analyzed for ZNF143 expression by immunoblotting. Consistent with the idea that ROS generation plays a role in the IGF-1 induction of ZNF143, H_2O_2 treatment increased ZNF143 expression in HCT116 cells (Figure 2D).

Receptor internalization is involved in IGF-1-induced ZNF143 expression in HCT116 cells

The internalization and recycling of IGF-1R has been demonstrated to mediate IGF-1-induced sustained signaling events, such as the phosphorylation of Akt, in glial progenitors (Romanelli *et al*., 2007), and IGF-1R internalization has been

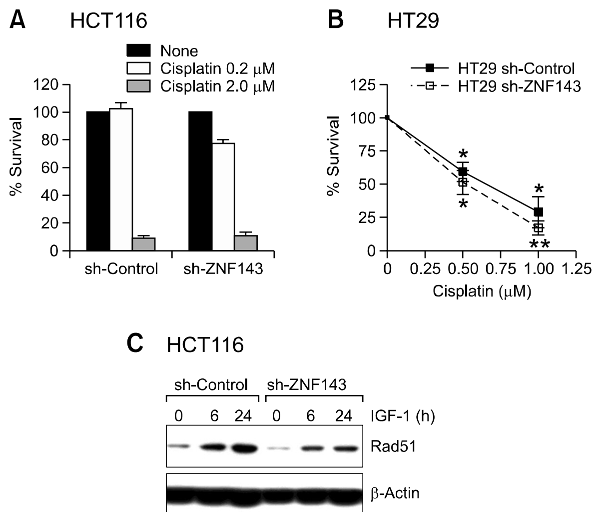

Figure 4. Knockdown of ZNF143 increases the sensitivity of HCT116 and HT29 colon cancer cells to cisplatin, and knockdown of ZNF143 reduces Rad51 expression in HCT116 cells. (A, B) Control and ZNF143 shRNA-expressing HCT116 (A) and HT29 (B) cells were incubated with cisplatin (0-2 μM) or buffer. The medium was replaced every 3 days until colonies formed. Stained colonies were counted under a microscope, and the numbers of colonies obtained were analyzed using GraphPad software. Data are expressed as means ± S.E. of at least three independent experiments. Statistical significance was assessed using paired Student's *t*-tests (*, $P < 0.05$, **, $P < 0.005$). (C) HCT116 sh-control, and HCT116 sh-ZNF143 cells were serum-starved, incubated with IGF-1 (100 ng/ml) for 0, 6, or 24 h, harvested, and analyzed for Rad51 expression by immunoblotting. Results shown are representative of at least three independent experiments.

shown to regulate signaling *via* the Shc/MAPK pathway but not the IRS-1 pathway (Chow *et al*., 1998). In addition, surface-localized receptor tyrosine kinases have been proposed to activate signaling pathways distinct from those activated by internalized, endosome-associated receptor tyrosine kinases (Jullien *et al*., 2002). Thus, the ligand-induced phosphorylation of Akt in IGF-1/IGF-1R signaling might be sustained through mediators activated by internalized, endosome-associated IGF-1R. Previously we and reported that ROS generation can be attenuated by blocking receptor internalization, in which NADPH oxidase plays a role in tumor necrosis factor α signaling in non-phagocytic cells (Woo *et al*., 2006; Kim *et al*., 2008). Thus, we hypothesized that a receptor internalization blockade might affect IGF-1-induced ROS generation.

To investigate the role of internalization of IGF-1R, serum-starved HCT116 cells were treated with IGF-1 for 6 h in the absence or presence of the internalization inhibitor monodansylcadavarine (MDC). When ZNF143 and phosphorylated Akt levels were analyzed by immunoblotting, we found that MDC attenuated both the induction of ZNF143 expression by IGF-1 (Figure 3A) and the induction of Akt phosphorylation by IGF-1 (Figure 3C) suggesting that IGF-1R internalization plays a role in the IGF-1/PI3-kinase/Akt signaling pathway leading to ZNF143 expression in HCT116 cells.

To investigate the relationship between internalization and ROS generation in IGF-1-stimulated cells, cells were serum-starved, incubated with IGF-1 in the absence or presence of MDC, and analyzed for ROS generation by flow cytometry. Whereas IGF-1 treatment increased ROS levels in control (DMSO-treated) cells, this increase was attenuated in MDC-treated cells (Figure 3B). These data show the involvement of the PI3-kinase/Akt pathway and ROS followed by receptor internalization in IGF-1 signaling to ZNF143 expression. Next we investigated whether the PI3-kinase/Akt pathways and ROS crosstalk to induce ZNF143 expression in IGF-1-stimulated cells. Serum-starved cells were treated with IGF-1 for 0, 10, or 60 min in the presence of DPI (an NADPH oxidase inhibitor), N-acetylcysteine (NAC; an ROS scavenger), or DMSO (vehicle), and Akt phosphorylation at residues Thr^{308} and Ser^{473} was analyzed by immunoblotting. The phosphorylation of Akt, especially at Thr^{308}, in response to IGF-1 was attenuated by DPI or NAC treatment at 10 min, and this attenuation was enhanced at 60 min (Figure 3D), implying that ROS mediate sustained ligand-induced phosphorylation of Akt in IGF-1/IGF-1R signaling. These data suggest that ROS generation is linked to receptor internalization and ZNF143 expression in IGF-1-stimulated HCT116 cells *via* crosstalk with the PI3-kinase/Akt pathway.

ZNF143 might be involved in cancer cell resistance to cisplatin

ZNF143 binds cisplatin-modified DNA and is upregulated in cisplatin-resistant ovarian cancer cells. Furthermore, knockdown of ZNF143 increases the sensitivity of cancer cells to cisplatin, implying a role for ZNF143 in cisplatin resistance in cancer cells (Ishiguchi *et al*., 2004; Wakasugi *et al*., 2007). Here, we investigated whether knockdown of ZNF143 expression would affect the cisplatin sensitivity of cancer cells. Toward this end, we performed clonogenic assays with HCT116 and HT29 cells. Cells were plated on a 6-well plate, exposed to cisplatin, maintained for 9-14 days, fixed, stained, and counted to determine cell survival. As shown in Figure 4A and B, HCT116 sh-ZNF143 and HT29 sh-ZNF143 cells did not survive as well as HCT116 sh-control and HT29 sh-control cells when they were exposed to 0.2 or 0.5 μM cisplatin, respectively. Thus, knockdown of ZNF143 expression increased the sensitivity of

these cancer cells toward cisplatin, similar to previously reported findings (Wakasugi *et al*., 2007). As shown in Figure 4C, knockdown of ZNF143 reduced IGF-1 induction of Rad51, a ZNF143 target protein important in DNA repair (Trojanek *et al*., 2003; Wakasugi *et al*., 2007) in HCT116 cells suggesting a role for ROS in the cellular response to IGF-1.

Taken together, our data show that IGF-1 upregulates the expression of ZNF143, a transcription factor implicated in drug resistance, for the first time and show that the induction of ZNF143 by IGF-1 is through a mechanism involving the PI3-kinase/Akt pathway and ROS generation. In addition, our data show that ROS generation in response to IGF-1 might occur *via* receptor internalization. However, many questions remain to be answered, including those concerning whether or not GIPC, a binding partner of IGF-1R participates in receptor internalization processes and whether GIPC regulates the generation of ROS in response to IGF-1. We are currently investigating how ZNF143 and its target genes regulate the DNA damage response in cancer cells treated with anticancer agents.

Methods

Materials

Dulbeccos' modified eagle medium (DMEM) and defined fetal bovine serum (FBS) were from Hyclone (Logan, UT). AG1024, diphenyleneiodonium (DPI), SB203580, wortmannin, SP600125 and PD98059 were purchased from Calbiochem (La Jolla, CA). Monodansylcadaverine (MDC) was from Sigma Chemical Co. (St. Louis, MO). IGF-1 was from R&D systems, Inc. (Minneapolis, MN). Mouse monoclonal antibodies specific for β-actin, ZNF143, rabbit polyclonal antibodies for IGF-1R, Rad51, and goat polyclonal antibody for GIPC were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies specific for phospho-Akt^{473}, phospho-Akt^{308}, phospho-ERK1/2, phospho-p38 kinase, phospho-JNK, Akt, ERK1/2, p38 kinase, JNK and HRP-conjugated anti-mouse and anti-rabbit antibodies were from Cell Signaling Technology Inc. (Beverly, MA). shRNA-lentiviral particles against human ZNF143 and control were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Cisplatin was purchased from Sigma-Aldrich Chemical Corp. (St Louis, MO).

Cell culture

The human colon carcinoma cell line HCT116, the human colorectal adenocarcinoma cell line HT29, and the human liver carcinoma cell line HepG2 were obtained from the American Type Culture Collection. HCT116 cells were maintained as monolayers in Dulbecco's modified Eagle medium (DMEM), HT29 cells were maintained in McCoy's 5A medium, and HepG2 cells were maintained in modified Eagle medium. All maintenance media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). All cells were grown at 37°C in a humidified 5% CO_2 atmosphere.

Isolation of RNA and reverse transcription-polymerase chain reaction

Cells (10^6 cells/ml) were grown in 60-mm-diameter plates for 24 h and starved for an additional 24 h. Then, cells were treated with 100 ng/ml of IGF-1 for the indicated times, and harvested. Total cellular RNA was extracted with TriZol (Sigma, St. Louis, MO) and dissolved in diethyl pyrocarbonate-treated water. The RNA was quantified by UV scanning, and samples (1 μg) were reversetranscribed at 42°C for 60 min in 20 μl buffer (10 mM Tris, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, and 1 mM each of dATP, dCTP, dGTP, and dTTP) in the presence of oligo (dT) primer. Hot-start PCR was used to increase the specificity of amplification. Thirty-five cycles of PCR amplification were used for ZNF143. The ZNF143 sense primer was 5’-CAGCATTCCATACTGCCTCA-3’ and the antisense primer 5’-GAGATGGCTGTTCTCCAAGC-3’ (GenBank Accession No.: NM_003442). The PCR product consisted of 141 bp. Twenty-one cycles of PCR amplification were used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH sense primer was 5’-GAGTCAACG-GATTTGGTCGT-3’ and the antisense primer 5’-TTG-ATTTTGGAGGGA-TCTCG-3’ (GenBank Accession No.: NM_002046), generating a 248 bp product. The PCR products were subjected to electrophoresis on 1.5% (wt/vol) agarose gels, and the resulting bands were visualized with ethidium bromide and photographed using the GelDoc program (Bio-Rad, Chicago, IL).

Short hairpin RNA (shRNA)-mediated silencing of human ZNF143 in HCT116 and HT29 cells

For stable lentivirus-mediated expression of shRNA specific for the ZNF143 genes in HCT116 and HT29 cells, cells were grown for 24 h, incubated with polybrene (5 μg/ml) for 1 h and then, infected with the lentiviral vector (approximately 1 molar ratio of infection). After 48 h, the medium was replaced and cells were grown for 1 day. The control cell lines HCT116-sh-control and HT29-sh-control and the cell lines stably expressing ZNF143 shRNA (HCT116-sh-ZNF143 and HT29-sh-ZNF143) were selected in 10 μg/ml puromycin dihydrochloride and maintained in growth medium containing 1 μg/ml puromycin dihydrochloride. To avoid clonal variation, the individual clones for each stable cell line produced by infection were pooled.

Measurement of intracellular ROS by flow cytometry

Cells (10^5 cells/ml) were grown in 6-well plate for 24 h and starved for an additional 24 h. Then, cells were treated with 100 ng/ml of IGF-1 for the indicated times, washed with warm PBS, trypsinized, and quickly analyzed for green fluorescence by flow cytometry as described previously (You *et al*., 2004). For ROS detection, 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, 10 μM) was

added 10 min before harvest, and analysis was done with a FACS-Calibur (Becton-Dickinson, Mountain View, CA, USA) by the NCC FACS operator. In some experiments, cells were pretreated with an inhibitor, monodansylcardavarine (MDC, 50 μM) or DMSO 30 min prior to IGF-1 treatment. The cells were sorted at approximately 500 cells/s using saline as the sheath fluid and a 488-nm argon laser beam for excitation. A two-parameter dot-plot of the side light scatter (SSC) and forward light scatter (FSC) of the population was analyzed, and the DCF fluorescence of 10,000 gated cells was measured using log amplification. The arithmetic geometric mean fluorescence channel (Geo MFC) was derived with CellQuest.

Immunoblotting

Protein samples were heated to 95°C for 5 min, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in 8 or 10% (w/v) acrylamide gels, and transferred to polyvinylidene difluoride membranes for 1 h at 350 mA using a Bio-Rad transfer unit (Bio-Rad Laboratories, Inc., Hercules, CA). Membranes were blocked for 1 h in Tris-buffered saline containing 0.01% Tween 20 (TBST) and 5% nonfat dried milk, incubated for 2 h with primary antibody in TBST containing 2% bovine serum albumin (BSA), and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody. The blots were developed using an enhanced chemiluminescence kit (West-ZOL® plus, Western Blot Detection System, Intron Biotechnology Inc., South Korea).

Clonogenic assay

Cells (200 cells/well) were seeded in to a 6-well plate, grown for 24 h, exposed to various doses of cisplatin (0-20 μM), and maintained for 9 days until colonies were formed. They were fixed and stained using a Diff-Quik Stain Kit (Sysmex corp, Japan). Stained colonies were counted under a microscope. Experiments were performed in triplicate.

Statistical analysis

All data are expressed as percentages of the control and shown as means ± S.E. Statistical comparisons between groups were made using paired *t* tests with Prism 5.0 statistical software (GraphPad Software Inc., San Diego, CA). Values of $P < 0.05$ were considered significant.

Supplemental data

Supplemental Data include two figures and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-10-03.pdf.

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