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WWOX Induces MEK2-Mediated Cell Death in a p53 Independent Manner

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WW domain-containing oxidoreductase WWOX that contains two WW domains in the N-terminus, which are involved in protein-protein interactions, and an alcohol dehydrogenase (ADH) domain is a putative tumor suppressor and a proapoptotic protein. It has been reported that WWOX gene is located at the common fragile site FRA16D in chromosome 16q23.3-24.1 and its expression is down-regulated in various cancer types.[1,2] Furthermore, aberrantly spliced *WWOX* mRNA forms that were not detected in normal cells were detected in cancer cell lines and primary breast tumors.[3] In addition, loss of WWOX expression resulted in resistance to apoptosis induced by tumor necrosis factor, staurosporine, UV, and p53 overexpression whereas overexpressed WWOX promoted apoptosis synergistically with p53.[4]

WWOX is known to participate in several physiological functions through interaction with several regulators, such as Dvl proteins for the regulation of Wnt-catenin pathway,[5] JNK and c-Jun for JNK signaling,[4,6] RUNX2 for osteoblast differentiation[7], and p53/p73 for apoptosis.[8,9]

Previous report showed that WWOX is a novel regulator in extracellular signal-regulated kinase (ERK) signaling through interaction with MEK2.[10] ERK signaling is important for the control of cell proliferation, migration, cell division, and differentiation.[11] ERK is phosphorylated and activated by MAP kinase kinases (MAP2Ks) such as MEK1/2. However, ERK activated by either MEK1 or MEK2 might have opposite roles in cell survival. MEK1/ERK signal enhances cell proliferation, whereas the MEK2/ERK induces growth arrest at the G_1/S boundary.[12]

Since the previous study showed that WWOX interacts with MEK2 and activates ERK pathway[10] and WWOX is a putative tumor suppressor, it implicates that the association of WWOX with MEK2 may have a negative role in cell growth.

In this study, it was investigated whether WWOX regulates MEK2-mediated cell growth. Since ERK activity is increased by WWOX,[10] cell viability assays were carried out to test whether WWOX regulates MEK2-induced proliferation of HEK 293 cells. HEK 293 cells were transiently co-transfected with FLAG-tagged WWOX wild-type (WT) and HA-tagged MEK2 expression plasmids. To enhance MEK2 activity and susceptibility to apoptosis, cells were treated with 0.5 mM H_2O_2 for 16 h prior to assay. WWOX-induced cell death was significantly increased in the presence of MEK2 while MEK2 alone did not induce cell death (Fig. 1),

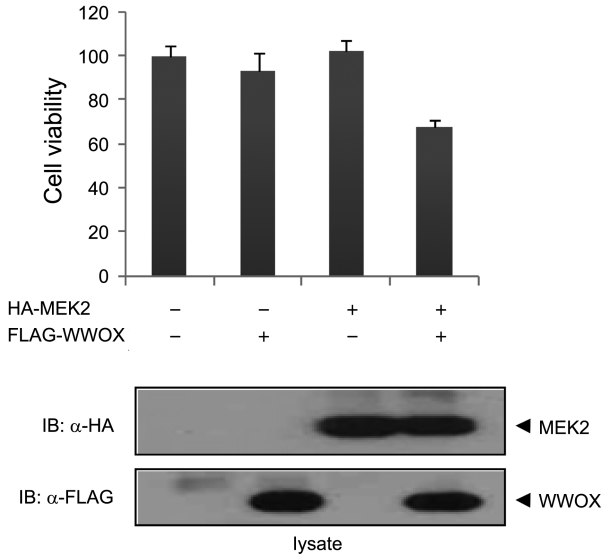

Figure 1. WWOX induces cell death in cooperation with MEK2. HEK 293 cells were transfected using HA-MEK2 or FLAG-WWOX WT expression plasmids. After 32 h of transfection, cells were treated with 0.5 mM H_2O_2 for 16 h and cell viability assays were carried out as described in the Experimental Section. Cell lysates were also prepared and then subjected to immunoblot analysis with an anti-HA or anti-FLAG antibody. The results presented are representative of three independent experiments. Error bars indicate ± SEM.

indicating that WWOX enhances MEK2-mediated cell death.

H_2O_2 decreases the cellular threshold for cell death induction by inducing pro-apoptotic events. The threshold concentration of H_2O_2 which affects WWOX/MEK2-mediated cell viability was investigated. HEK 293 cells co-transfected with FLAG-WWOX WT and HA-MEK2 expression plasmids were treated with various concentrations of H_2O_2 for 16 h prior to viability assays. Cells transfected with WWOX or MEK2 alone did not show any change of cell viability in the absence of H_2O_2 and those treated with 1 mM were severely decreased in cell viability (Fig. 2(a)). At 0.5 mM H_2O_2, cells transfected with both WWOX and MEK2 showed significantly decreased level of cell viability. These data suggest that WWOX is involved in cell death.

Since p53 is a key regulator of apoptosis induced by WWOX,[9] human colon epithelial HCT116 and HCT116 $p53^{-/-}$ cells were used for cell viability assays to test whether p53 is critical in WWOX/MEK2-mediated cell death (Fig. 2(b)). The results implicate that p53 is dispensable for

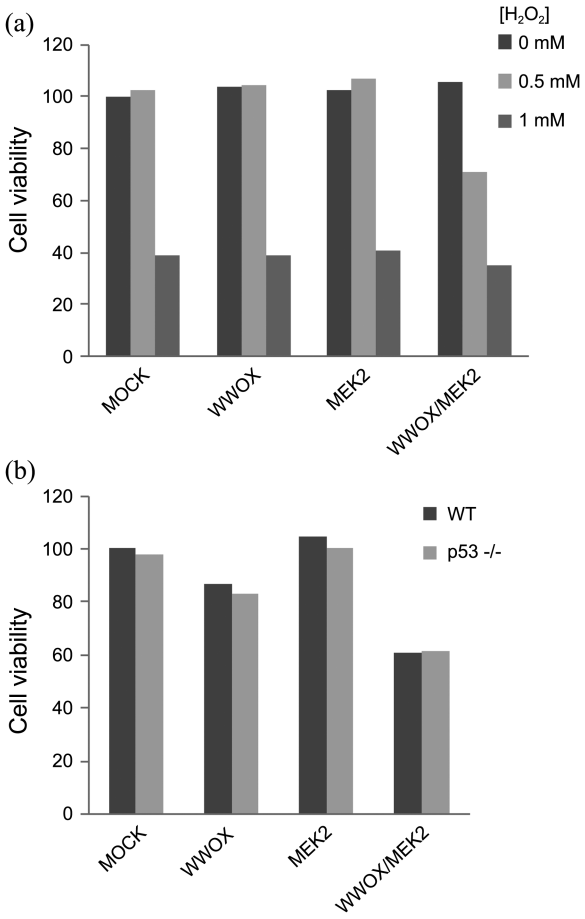

Figure 2. WWOX induces MEK2-mediated cell death independent of p53 upon exposure to H_2O_2. (a) HEK 293 cells were transfected with HA-MEK2 or FLAG-WWOX WT expression plasmids. After 32 h of transfection, cells were treated with 0, 0.5, or 1.0 mM H_2O_2 for 16 h and cell viability assays were carried out as described in the Experimental Section. To minimize the influence of transfection reagents, mock-transfected cells (MOCK) were considered 100% viable. Three independent experiments were performed with similar results. (b) HCT116 and HCT116 $p53^{-/-}$ cells were transfected with HA-MEK2 or FLAG-WWOX WT expression plasmids. After 40 h of transfection, cells were treated with 0.5 mM H_2O_2 for 8 h and cell viability assays were carried out as described in the Experimental Section. Mock-transfected cells (MOCK) were considered 100% viable.

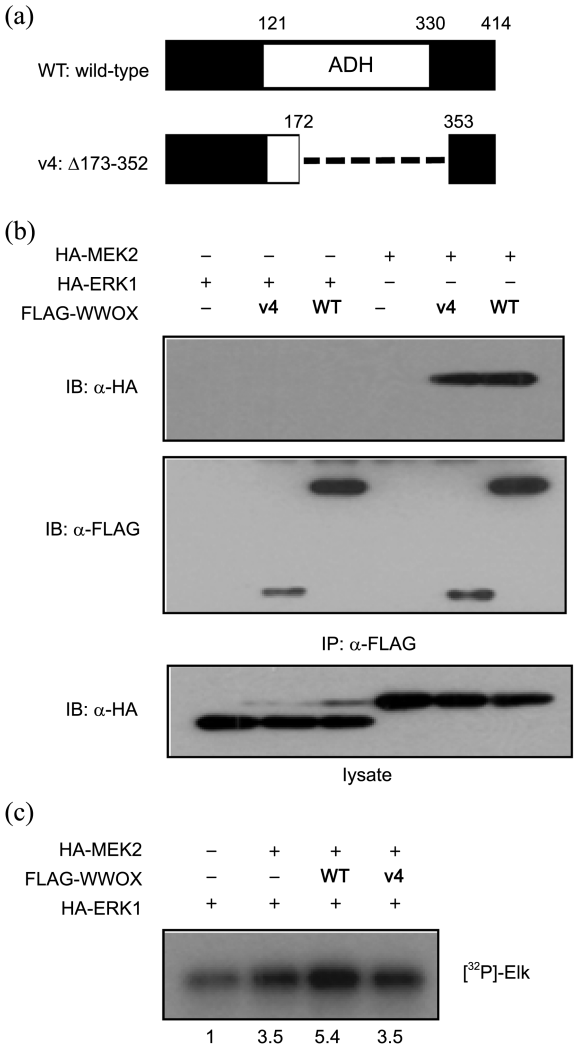

Figure 3. Alcohol dehydrogenase (ADH) domain of WWOX is important for MEK2-mediated ERK activity. (a) Schematic structures of WWOX WT and a variant v4. The amino acid sequence (414 amino acids) of WWOX possesses an alcohol dehydrogenase (ADH) domain (amino acids 121-330). v4 has a deletion in the ADH domain. (b) HEK 293 cells were transfected using HA-MEK2, HA-ERK, or FLAG-WWOX expression plasmids. After 48 h of transfection, cell lysates were prepared and then immunoprecipitated with anti-FLAG M2-agarose. Immunoprecipitates were subjected to immunoblot analysis with an anti-HA antibody as described in the Experimental Section. (c) HEK 293 cells were transfected with 1 μg of plasmids as indicated. After 48 h of transfection, cells were lysed and immunoprecipitated with anti-HA agarose. ERK activities were determined by *in vitro* kinase assays. *In vitro* kinase activities are shown as -fold increase relative to that of HA-ERK1 protein from transfected cells.

WWOX/MEK2-mediated cell death, suggesting that MEK2 activated by WWOX enhances death signals other than p53 pathway.

WWOX possesses an alcohol dehydrogenase (ADH) domain in the central region of the protein. It was investigated whether ADH domain is involved in the regulation of MEK2 activation. An alternatively spliced form of WWOX, v4, was used for this investigation since the isoform has deletion of most ADH domain (Fig. 3(a)). The v4 form of WWOX was used for binding assays with MEK2 and *in vitro* kinase assays using GST-Elk as a substrate of ERK that is a downstream factor of MEK2 (Fig. 3(b) and 3(c)). The results show that the v4 isoform interacts with MEK2 but does not activate MEK2 activity, when compared to WWOX wild-type.

In conclusion, the results of this study suggest that WWOX acts as a positive regulator of cell death *via* activating MEK2 kinase activity. HEK 293 cells overexpressing both WWOX and MEK2 are susceptible to cell death at 0.5 mM of H_2O_2. It is also shown that p53 is not involved in WWOX/MEK2-induced cell death. Furthermore, the ADH domain of WWOX is indispensable for activation of MEK2.

Experimental Section

Cell Culture and Transfection. Human colon epithelial HCT116, HCT116 $p53^{-/-}$, and human embryonic kidney (HEK) 293 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin in the presence of 5% CO_2. For transient transfection, 1.4×10^6 cells were plated in each 60-mm cell culture plate, grown overnight, and transfected with DNA using LipofectAMINE (Invitrogen).

Plasmid Constructs. HA-MEK2, HA-ERK1, FLAG-WWOX WT, and FLAG-WWOX v4 (173-352) for expression in mammalian cells were constructed by polymerase chain reaction (PCR), followed by cloning into the pcDNA3.1/Zeo plasmid (Invitrogen). GST-Elk was constructed in the pGEX-6P-1 (Amersham Biosciences, Little Chalfont, UK) plasmid for protein expressions in *Escherichia coli*.

Reagents and Antibodies. Anti-HA antibody and anti-HA agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-FLAG M2 antibody and anti-FLAG M2 agarose beads were purchased from Sigma-Aldrich (St. Louis, MO).

Immunoblotting Analysis. After HEK 293 cells were transiently transfected with FLAG- or HA-tagged expression plasmids for 48 h, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in a lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/mL aprotinin for 30 min at 4 °C. Cleared cell lysates from centrifugation were resuspended with protein sample buffer, boiled at 100 °C for 5 min, subjected to SDS-PAGE, and subsequently transferred onto nitrocellulose membrane. Immunoblotting was carried out as previously described.[13]

***In vitro* Binding Assays.** Cells were co-transfected with HA-MEK2 and FLAG-WWOX expression plasmids. After 48 h of transfection, cells were washed twice with phosphate-buffered saline (PBS) and extracted in the lysis buffer. Cell extracts were clarified by centrifugation, and the supernatants were incubated with anti-FLAG M2-agarose for 5 h at 4 °C with rotation. After binding, the beads were collected by centrifugation at 6,000 rpm for 2 min and washed five times with lysis buffer. The bound proteins were eluted with the SDS-PAGE sample buffer, separated by SDS-PAGE, and then immunoblotted with an anti-HA antibody. The protein bands were visualized using the ECL detection system (Pierce, Rockford, IL, USA).

***In vitro* Kinase Assays.** For the immune complex kinase assay, HEK 293 cells were transfected with appropriate expression plasmids. After 48 h of transfection, cells were lysed in the lysis buffer. Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with anti-HA agarose beads for 16 h at 4 °C. The beads were washed once with lysis buffer, twice with a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, and 1 mM PMSF, and then once with a solution containing 20 mM Tris-HCl (pH 7.5) and 20 mM $MgCl_2$. The beads were then resuspended in 20 μL of kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol) containing 20 μM ATP and 0.3 μCi of [γ-^{32}P] ATP with 1 μg of GST-Elk for 30 min at 30 °C. The products of kinase reactions were separated by SDS-PAGE. The gels were dried and exposed to film.

Cell Viability Assay. Cell viability was determined using a CCK-8 cell viability assay kit (DOJINDO Laboratories, Japan). For transfected HEK 293 cells, cells (5×10^3 cells/well) were cultured in DMEM medium supplemented with 5% FBS and incubated for 32 h in a 96-well plate. Cells were treated with 0.5 mM H_2O_2 for 16 h prior to assay. For HCT116 cells, cells were treated with 0.5 mM H_2O_2 for 8 h prior to assay. 10 μL of cell viability assay kit solution was added to each well of the plate. After incubation for 1 h at 37 °C in the dark, absorbances were measured at 450 nm using a multiwell plate reader.

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