The Requirement of c-Jun N-terminal Kinase 2 in Regulation of Hypoxia-inducing Factor-1α mRNA Stability*

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Dongyun Zhang‡, Jingxia Li‡, Min Zhang‡, Guangxun Gao‡, Zhonghong Zuo‡, Yonghui Yu‡, Linda Zhu‡, Jimin Gao‡, and Chuanshu Huang‡‡

From the ‡Nelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, New York 10987 and the §Zhejiang Province Key Laboratory of Medical Genetics, School of Life Science, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China

Background: Messenger RNA of hif-1α could be regulated by post-transcriptional mechanisms.

Results: Depletion of either JNK2 or nucleolin affected hif-1α mRNA stability.

Conclusion: JNK2 regulated nucleolin expression and might in turn stabilize hif-1α mRNA.

Significance: We provided more evidence for the oncogenic roles of JNK2 and nucleolin in regulating the cancer microenvironments by controlling HIF-1α expression.

The mRNA of hif-1α is considered as being constitutively and ubiquitously expressed, regardless of the level of oxygen tension. However many recent reports have showed that hif-1α mRNA could be regulated by natural antisense transcripts, potential microRNAs, and low O2. In this study, it was found that a deficiency of JNK2 expression reduced HIF-1α protein induction in response to nickel treatment resulting from the impaired expression of hif-1α mRNA. Both the promoter luciferase assay and mRNA degradation assay clearly showed that depletion of JNK2 affected stability of hif-1α mRNA, rather than regulated its transcription. In addition, nucleolin, a classic histone chaperone, was demonstrated to physically bind to hif-1α mRNA and maintain its stability. Further investigation indicated that JNK2 regulated nucleolin expression and might in turn stabilize hif-1α mRNA. Collectively, we provided one more piece of evidence for the oncogenic role of JNK2 and nucleolin in regulating the cancer microenvironments by controlling HIF-1α expression.

Nickel is a widely distributed natural metal, and humans are constantly exposed to environmental nickel because of its constitutive release from natural sources, or pollutants from nickel manufacturing industries or airborne particles from combustion of fossil fuels (1). The causality between nickel exposure and cancer development has been supported by both animal studies and epidemiologic investigations (2). It was reported that both the genetic and epigenetic factors were attributable to the carcinogenic characteristics of mode of action for nickel (3, 4). Induction of hypoxia-inducible factor-1α (HIF-1α) by nickel treatment was documented both in vivo and in vitro (5). The reports from our group and others have demonstrated the requirement of HIF-1α for the malignant transformation caused by nickel exposure in cultured cell system (6–8). However, the molecular mechanisms about the regulation of HIF-1α expression after nickel exposure are far from fully understood.

HIF-1 is a heterodimeric transcription factor, and plays a key role in cellular adaptations to a deficiency of oxygen supply by controlling expressions of a series of genes involved in angiogenesis, oxygen transport, and glucose metabolism (9). HIF-1 consists of an oxygen-regulated HIF-1α subunit and a constitutively expressed hydrocarbon receptor nuclear translocator (also called HIF-1β or ARNT) (7). Dysregulation of HIF-1α occurs in renal cell carcinoma, breast, lung, and ovarian cancers, with a strong correlation to tumor metastasis and a poor prognosis for patients (10). HIF-1α protein is very dynamic in normoxia, and is normally regulated by post-translational mechanism. In contrast, hif-1α mRNA is constitutively and ubiquitously expressed, regardless of the level of oxygen tension (11). However, an increasing number of recent studies have shown that hif-1α mRNA could be regulated by natural anti-sense transcripts (12, 13), potential microRNAs (14, 15), and low O2 pressure in the renal medulla in rats (16) and sea bass (17). The hif-1α mRNA contains 823 bp long 3′-UTR that is AU-rich and contains AUUUA pentamers that are conserved ubiquitously, regardless of the level of oxygen tension (11). However, an increasing number of recent studies have shown that hif-1α mRNA could be regulated by natural anti-sense transcripts (12, 13), potential microRNAs (14, 15), and low O2 pressure in the renal medulla in rats (16) and sea bass (17). The hif-1α mRNA contains 823 bp long 3′-UTR that is AU-rich and contains AUUUA pentamers that are conserved among rat, mouse, and human (15, 16). It is now known that a group of RNA-binding proteins can bind to AU-rich elements (ARE) in 3′-UTR of mRNA and regulate the mRNA turnover rate (60). In studies reported here, it was found for the first time that nucleolin could physically bind to and stabilize hif-1α mRNA in cell culture system.

Nucleolin (also known as C23) is a multifunctional phosphoprotein whose expression is abundant, i.e. it represents up to 5–10% of nucleolar proteins in exponentially-growing cells (18). Nucleolin is also implicated in several pathologies including viral infection (19), autoimmune diseases (20), Alzheimer

DMOG, dimethyloxaloylglycine; HRE, hypoxia-responsive element; PHD, prolyl hydroxylase domain; Act, actinomycin.
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disease (21), Parkinson disease (22), and cancer development (40). The role of nucleolin in these pathological processes roots from its function(s) in DNA metabolism, chromatin re-modeling, ribosome biogenesis and mRNA decay (23). Nucleolin has been found to bind to the mRNA of several important genes, including p53 (24), bcl-2 (25), and bcl-xl (26), leading to regulation on mRNA turnover or translation. Moreover, nucleolin has been shown to have binding activity to a number of G-rich oligonucleotides, like the G-quadruplex structure in the promoter region of c-myc gene (27) and vegf gene (28). Gonzalea et al. showed, in an in vitro filter binding assay, that nucleolin bound to the G-quadruplex structure in 5'-UTR of hif-1α mRNA (27). However, it is still open for investigation as to whether or not nucleolin could regulate HIF-1α expression in vivo. Therefore, the goal of this study, reported here, was to obtain experimental evidence for this issue in a cell culture model.

MATERIALS AND METHODS

Cell Culture and Reagents—Mouse embryonic fibroblasts (MEFs), including WT cells and JNK2−/− cells (29) as well as their stable transfectants, mouse fibroblast NIH3T3 cells, and human embryonic kidney cells HEK293T were cultured in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Nova-Tech, Grand Island, NE). The MEFs were immortalized according to 3T3 protocol (30). Tristetraprolin (TTP) knock-out MEFs and WT MEFs (31) were kindly provided by Dr. Perry J. Blackshear (National Institute of Environmental Health Sciences). The cells were cultured in DMEM supplemented with 10% FBS, and identified by PCR, using the primers of 5′-CTG AGC TGT CAC CCT CAC CT-3′ and 5′-TGG TGC TGG GGG TAG TAG AC-3′.

Actinomycin D was purchased from Calbiochem. Cordycepin was purchased from Santa Cruz Biotechnology Inc. Nickel chloride was purchased from Sigma-Aldrich. Dimethyloxyglycine (DMOG) was purchased from Frontier Scientific (Logan, UT). A protein phosphatase was purchased from New England Biolabs (Ipswich, MA). Antibodies against HIF-1α (for Western blotting) and HIF-1β were purchased from Novus Biologicals, Inc. (Littleton, CO); Anti-JNK1 antibody was from Invitrogen (Carlsbad, CA); Antibodies against non-phosphorylated c-Jun, JNK1/2, phosphor-c-Jun at Ser-73, phosphor-JNK at Thr-183/Tyr-185 were purchased from Cell Signaling Technology (Beverly, MA); anti-β-actin, α-tubulin, HA, nucleolin, and HIF-1α (for immunoprecipitation) antibodies were purchased from Sigma; Anti-GFP antibody was purchased from Santa Cruz Biotechnology.

Constructs and Transfections—The HRE luciferase reporter was constructed by inserting the sequence of the HIF-1α binding site into the luciferase reporter vector pG12-basic, as previously described (32, 33). The vegf-luciferase reporter was constructed by inserting a 2.65-kb KpnI-BssHII fragment of the human vegf promoter sequence from −2274 to +379, relative to the transcription initiation site into the pGL2-basic vector (Promega, Madison, WI) (32, 33). Each of the above reporters (2 μg) was stably transfected into WT MEFs and JNK2−/− cells in combination with hygromycin-resistance plasmid (0.8 μg) for positive clone selection. HA-JNK2 expression construct (2 μg) (34) in combination with hygromycin-resistance plasmid (0.8 μg) was transfected into JNK2−/− cells. The stable transfectants, named as JNK2−/− (HA-JNK2), were established by hygromycin selection (400 μg/ml) and all transfectants were pooled as mass culture as described in our previous publications (35–37). The shRNA sets for Nucleolin and JNK2 were purchased from Open Biosystems (Thermo Fisher Scientific, Huntsville, AL), and were transfected stably into WT MEFs or NIH3T3 cells, respectively. The hif-1α promoter luciferase reporter (PH800) was kindly provided by Dr. Carine Michiels (Laboratory of Biochemistry and Cellular Biology, FUNDP-University of Namur, Belgium) (38). The Nucleolin promoter luciferase reporter was kindly provided by Dr. Bruno Amati (Cellular Growth Control Unit, Swiss Institute for Experimental Cancer Research, Switzerland) (39). GFP-nucleolin expression vector was kindly provided by Dr. Michael B. Kastan (Comprehensive Cancer Center, St. Jude Children’s Research Hospital, Memphis, TN) (29).

RT-PCR—Total RNA was extracted from the cells using Trizol reagent (Invitrogen). Total cDNAs were synthesized by ThermoScript ™ RT-PCR system (Invitrogen). The primers for mouse hif-1α were: 5′-AGC CCT AGA TGG CTT TGT GA-3′ and 5′-TAT CGA GGC GTG TGT GAC TG-3′; for mouse nucleolin were 5′-GGA GGT TGT CAT CCC TCA GA-3′ and 5′-TCC TCC TCA GCC ACA CTC TT-3′; for mouse ahif-1α were 5′-GCT GGA AGG TTT GTG GTG TT-3′ and 5′-TAG GAA TGA TGG GCC GGC ATT TA-3′; for mouse β-actin were 5′-CAT CGG TAA AGA ATG TGC CTA CTC G-3′ and 5′-ACC CAG CTC AGT AAC AGT CC-3′ (also used in real-time PCR). The PCR products were separated over 2% agarose gels, stained with ethidium bromide. The results were imaged with Alpha Innotech SP image system (Alpha Innotech Corporation, San Leandro, CA). The densitometric analyses of the product bands were conducted using the software of ImageQuant 5.2 (GE Healthcare). The results shown were representative of three independent experiments.

Quantitative RT-PCR—The same cDNAs that were used for the above RT-PCR were also analyzed for real-time PCR using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The primers for real-time PCR were: mouse hif-1α 5′-GAA GAC AAC GCG GGC ACC GA-3′ and 5′-TGC TTC GCC GAG ATC TTG CTG C-3′; mouse nucleolin 5′-GAG GAC CCC CTT CGT CGC CT-3′ and 5′-GCC TCA CCG TGG GTT TTG CCA-3′. The real-time PCR was conducted following the protocol for Fast SYBR Green Master Mix kit (Applied Biosystems). Briefly, an initial activation was performed at 95 °C for 20 s, followed by 40 cycles of denaturation at 95 °C for 1 s, and annealing and extension at 60 °C for 20 s. The relative mRNA levels were obtained by exponentially transforming ΔCt values to 2ΔΔCt and the mean values were calculated with one standard deviation (41). The data were representative of three independent experiments.

Luciferase Reporter Assay—The cells transfected with the HRE, vegf, hif-1α, or nucleolin promoter luciferase reporters were seeded into 96-well plates (8 × 103/well) and were subjected to the specific treatments when cultures reached 70 to 80% confluence. pRL-TK vector was used as an internal control. Cellular lysates were prepared, and luciferase activities were
determined using a luminometer (Wallac 1420 Victor 2 multi-label counter system), as previously described (42).

Western Blotting—MEFs and their transfectants were plated in 6-well plates and cultured in normal 10% FBS DMEM until 70–80% confluence. After various treatments, the proteins were extracted and total protein was quantified with a Dc protein assay kit (Bio-Rad). Western blotting was carried out as previously described (43). Primary antibody-bound proteins were detected by using an alkaline phosphatase-linked secondary antibody and an ECF Western blotting system (Amersham Biosciences, Piscataway, NJ).

Pulse Assays—Cells were exposed to nickel (0.5 mm) for 12 h, then incubated with methionine-cysteine-free DMEM (Invitrogen) containing 2% dialyzed fetal calf serum (Invitrogen) for 1 h. 35S-labeled methionine/cysteine (Trans 35S-Label; ICN) (250 μCi/dish) was added, and the cells were cultured for the time periods indicated. The cells were collected with lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na3VO4, 0.5% Nonidet P-40, and complete protein inhibitors mixture tablet) on ice. Total lysate of 500 μg was incubated with 2 μg of anti-HIF-1α monoclonal antibody (Sigma) or control IgG antibody for negative control for 2 h at 4 °C. Then protein-A/G plus-agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that were pre-cleared by 20 mg/ml BSA were added into the mixture and incubated with agitation for an additional 2 h at 4 °C. The immunoprecipitated samples were washed with the cell lysis buffer 5 times and heated at 100 °C for 5 min. Radiolabeled immunoprecipitated samples were washed with the cell lysis buffer 5 times and heated at 100 °C. The immunoprecipitated samples were washed with the cell lysis buffer 5 times and heated at 100 °C for 5 min. Radiolabeled HIF-1α protein as well as the input whole cell lysate (WCL) were assessed using SDS-polyacrylamide gels (10%) analysis.

Dephosphorylation Assay—After exposure to the specific nickel treatment, cells were collected by centrifuge, resuspended in PBS, and homogenized by sonication. About 50 μg of whole cell lysate was used as substrate and incubated with λ phosphatase (New England Biolabs) for 40 min at 30 °C. Dephosphorylated protein as well as the input whole cell lysate (WCL) were assessed using SDS-polyacrylamide gels (10%) analysis.

RNA-IP—The cells were cultured in 10-cm dishes and harvested by scraping after the specific treatment. Polysome lysis buffer (PLB), containing: 10 mM HEPES pH 7; 100 mM KCl; 5 mM MgCl2; 25 mM EDTA; 0.5% IGEPAI; 2 mM DTT; 50 units/ml RNase OUT; 50 units/ml Superase IN; 0.2 mg/ml heparin; and complete proteinase inhibitor, was used to lyse the cell pellet. The cell lysate was centrifuged at 14,000 × g for 10 min at 4 °C. The anti-nucleolin antibody and agarose beads A/G were added into the supernatant and rotated overnight at 4 °C in NET2 buffer containing: 50 mM Tris-HCl; pH 7.4; 150 mM NaCl; 1 mM MgCl2; 0.05% IGEPAI; 50 units/ml RNase OUT; 50 units/ml Superase IN; 1 mM dithiothreitol; and 30 mM EDTA. The beads were washed three times, resuspended in 100 μl of NET2 and 100 μl of SDS-TE (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 2% SDS) then incubated for 30 min at 55 °C, with occasional mixing. The RNAs in the buffer were extracted by phenol-chloroform-isooamyl alcohol. RT-PCR was performed to detect the mRNA present in the immune-complex.

RNA Pull-down Assay—The 3′-UTR of mouse hif-1α mRNA (NM_010431.2) was amplified by PCR from cDNA of WT MEFs, using the pair of primers (forward: 2994-TTG GGT TTT TGT TTC TGT TGG-3014; reverse: 4125-TTT CCT GGT CCA CAG AAG ATG-4105). The PCR product was used for in vitro transcription in the presence of biotinylated-UTP (MAXIscript® Kit, Ambion, Inc., Grand Island, NY). The biotinylated RNAs were incubated with cell lysis from WT MEFs (in PLB buffer) in a final volume of 200 μl at room temperature for 30 min and captured by streptavidin MagneSphere Paramagnetic Particle (Promega). After washing according to the manufacturer’s instructions, bound proteins were eluted in Laemml buffer and resolved by SDS-PAGE, electrically transferred to PVDF membrane, and the presence of nucleolin and HIF-1α was detected with Western blotting assay (44).

Statistical Analysis—The significance of the difference between the treated and untreated groups, or between different cell lines, was determined with the Wilcoxon Rank Sum Test. The results are expressed as mean ± S.D. A p value of ≤ 0.05 was used to assign significance.

RESULTS

JNK2 Regulated HIF-1α Protein Expression—Although it is well documented that JNKs can regulate the activity of dozens of transcription factors (45), there is no report about the relationship between JNKs and HIF-1α. Our previous findings show that JNK1 is responsible for HIF-1α expression through maintaining its protein stability (7). Therefore, it is of interest to investigate whether JNK2 also plays a role in the regulation of HIF-1α expression and whether it holds the distinct underlying molecular mechanisms from JNK1. To explore this, we first compared HIF-1α protein levels among WT(vector), JNK2−/− (vector) and JNK2−/− (HA-JNK2) MEFs following nickel treatment. The results showed that depletion of JNK2 expression in MEFs attenuated HIF-1α protein accumulation upon nickel treatment at all time points and doses tested, whereas the expression level of HIF-1β (the constitutively expressed subunit of HIF-1) was comparable between JNK2−/− and WT cells (Fig. 1A). We then evaluated the HIF-1α-dependent transactivation activity by transfecting a construct containing a hypoxia-responsive element (HRE) luciferase reporter into both WT and knock-out cells. The HRE luciferase activity was significantly increased in WT MEFs following nickel exposure; however, it was attenuated in JNK2−/− cells (Fig. 1, B and C), indicating that both HIF-1α protein expression and its activity were impaired in JNK2−/− cells following nickel exposure. We also determined the transcriptional induction of vascular endothelial growth factor (vegf), a well-known downstream target gene of HIF-1. As expected, the induction of vegf transcription was decreased in JNK2−/− cells as compared with that in WT cells (Fig. 1D).

To further confirm whether the impairment of HIF-1α protein induction in JNK2−/− cells was directly due to jnk2 gene deficiency rather than changes of other genes during the establishment of immortalized cell lines, primarily cultured MEFs (at passage 7) were utilized. Consistent with the results observed in immortalized MEFs, HIF-1α protein accumulation following nickel exposure was dramatically impaired in primary JNK2−/− cells when compared with that in WT cells at the same passage (Fig. 2A). This effect was reproduced in another mouse fibroblast cell line NIH3T3, by introducing two different
shRNAs of JNK2 to knockdown endogenous JNK2 expression (Fig. 2B). Also, the reconstitution of JNK2 in JNK2−/− cells restored HIF-1α protein expression upon nickel exposure (Fig. 1A).

The mammalian prolyl hydroxylase domain (PHD) enzymes are critical for VHL-dependent HIF-1α proteasomal degradation (11, 46). Derivation of oxygen, transition metals (cobalt and nickel), and 2-oxoglutarate inhibitor (dimethyloxalylglycine, DMOG), all impair the activity of PHD enzymes. In the present studies, the hypoxia as well as several chemicals that mimic hypoxia conditions were used to investigate the accumulation of HIF-1α protein in WT(Vector), JNK2−/− (Vector) and JNK2−/− (HA-JNK2) cells. As shown in Fig. 2, C and D, the induction of HIF-1α protein was impaired upon deletion of the JNK2 gene in all the above experimental conditions, while it could be restored in JNK2−/− (HA-JNK2) cells. Therefore, it was concluded that JNK2 might play a crucial role in HIF-1α protein accumulation under hypoxic conditions.

**JNK2 Expression Was Crucial for Maintaining hif-1α mRNA Stability**—As observed in the above studies and other findings (47), induced HIF-1α protein migrated with a diffused pattern in SDS- polyacrylamide gels, suggesting HIF-1α protein undergoes strong post-translational modifications. It is known that protein phosphorylation can markedly modify the migration
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A and B, WT (A) or WT and JNK2−/− (B) MEFs (8 × 10⁶/well) were seeded into 10-mm dish and exposed to 0.5 μM nickel for 12 h and 24 h (A), or 24 h (B). The whole cell lysate was incubated with λ phosphatase for 40 min at 30 °C. Proteins were resolved in SDS-PAGE and revealed by Western blotting assay. C, WT(Vector), JNK2−/− (Vector), and JNK2−/− (HA-JNK) MEFs were treated with nickel (0.5 mM) for 12 h in complete medium. Cells were then accommodated in methionine- and cysteine-free DMEM for 1 h. 35S-labeled methionine and cysteine was then added for the indicated times for pulse assay. Cell extracts were immunoprecipitated with anti-HIF-1 antibody or control IgG, and subjected to SDS-PAGE. Autoradiography was used to visualize 35S-labeled HIF-1α. D, hif-1α mRNA levels in the individual cells were determined by real-time PCR. The asterisk (*) indicates a significant decrease as compared with those in WT(Vector) and JNK−/− (HA-JNK2) MEFs or non-silencing control cells (p < 0.05). E, basal levels of hif-1α promoter activity were evaluated by transfecting the indicated cells with a construct containing hif-1α promoter-driven luciferase. pRL-TK vector was used as an internal control. The results are expressed as the ratios of firefly to Renilla luciferase activity, as means ± S.D. (n = 3). The asterisk (*) indicates a significant increase as compared with that of WT(Vector) cells or JNK−/− (HA-JNK) cells (p < 0.05). F and G, mRNA degradation rate of hif-1α was detected following treatment with actinomycin D (5 μM) for the indicated time. The PCR products were separated over 2% agarose gels, stained with ethidium bromide. The densitometric analyses of the product bands were conducted using the software of ImageQuant 5.2 (GE Healthcare). The results were shown as means ± S.D. (n = 3). H, indicated cells were treated with cordycepin (5 μM) for 4 h. Real-time PCR was conducted to detect the hif-1α mRNA expression. The asterisk (*) indicates a significant decrease as compared with that in WT cells under the same treatment (p < 0.05).

FIGURE 3. JNK2 regulated hif-1α mRNA stability. A and B, WT (A) or WT and JNK2−/− MEFs (8 × 10⁶/well) were seeded into 10-mm dish and exposed to 0.5 μM nickel for 12 h and 24 h (A), or 24 h (B). The whole cell lysate was incubated with λ phosphatase for 40 min at 30 °C. Proteins were resolved in SDS-PAGE and revealed by Western blotting assay. C, WT(Vector), JNK2−/− (Vector), and JNK2−/− (HA-JNK) MEFs were treated with nickel (0.5 mM) for 12 h in complete medium. Cells were then accommodated in methionine- and cysteine-free DMEM for 1 h. 35S-labeled methionine and cysteine was then added for the indicated times for pulse assay. Cell extracts were immunoprecipitated with anti-HIF-1 antibody or control IgG, and subjected to SDS-PAGE. Autoradiography was used to visualize 35S-labeled HIF-1α. D, hif-1α mRNA levels in the individual cells were determined by real-time PCR. The asterisk (*) indicates a significant decrease as compared with those in WT(Vector) and JNK−/− (HA-JNK2) MEFs or non-silencing control cells (p < 0.05). E, basal levels of hif-1α promoter activity were evaluated by transfecting the indicated cells with a construct containing hif-1α promoter-driven luciferase. pRL-TK vector was used as an internal control. The results are expressed as the ratios of firefly to Renilla luciferase activity, as means ± S.D. (n = 3). The asterisk (*) indicates a significant increase as compared with that of WT(Vector) cells or JNK−/− (HA-JNK) cells (p < 0.05). F and G, mRNA degradation rate of hif-1α was detected following treatment with actinomycin D (5 μM) for the indicated time. The PCR products were separated over 2% agarose gels, stained with ethidium bromide. The densitometric analyses of the product bands were conducted using the software of ImageQuant 5.2 (GE Healthcare). The results were shown as means ± S.D. (n = 3). H, indicated cells were treated with cordycepin (5 μM) for 4 h. Real-time PCR was conducted to detect the hif-1α mRNA expression. The asterisk (*) indicates a significant decrease as compared with that in WT cells under the same treatment (p < 0.05).

pattern in SDS-polyacrylamide gels. Therefore, we evaluated whether the diffused band of HIF-1α protein was due to phosphor-modification. As shown in Fig. 3A, incubation of whole cell lysate (WCL) with a nonspecific protein phosphatase, λ phosphatase, dramatically converted the diffused HIF-1α protein band to a clear sharp band on the same gel. This result suggested that HIF-1α was phosphorylated in vivo and that this kind of modification was responsible for the reduced mobility on SDS-polyacrylamide gel.

To answer whether the decreased HIF-1α protein induction in JNK2−/− cells was due to less protein synthesis or due to impaired phosphorylation, the dephosphorylated form of HIF-1α protein was compared in WT and JNK2−/− cells in the presence of λ phosphatase. As shown in Fig. 3B, the dephosphorylated form of HIF-1α protein was obviously reduced in JNK2−/− cells when compared with that in WT cells. This result indicated that the reduction of the modified form of HIF-1α protein observed in JNK2−/− cells was caused by impairment of nascent HIF-1α protein synthesis rather than a defect of post-translational modification. Pulse analysis was conducted in turn, using 35S-labeled methionine and cysteine to monitor HIF-1α synthesis rate. It was found that newly translated HIF-1α protein was observed at 10 min after addition of labeling medium, and gradually increased until 60 min in WT(Vector) and JNK2−/− (HA-JNK2) cells. However, in JNK2−/− (Vector) cells HIF-1α protein translation amount was dramatically reduced (Fig. 3C), suggesting that de novo HIF-1α protein synthesis was defective in JNK2−/− (Vector)
cells. It was noted that the background in JNK2−/− (HA-JNK2) lanes of Coomassie Blue staining was slightly higher than those of the JNK2−/− (Vector) lanes (Fig. 3C). Thus, we anticipated this might be due to the photographing process. The explanation was supported by our data showing that the bands of 35S-labeled WCL in JNK2−/− (HA-JNK2) are slightly weaker in comparison to those in JNK2−/− (Vector) cells, however the de novo HIF-1α protein bands in JNK2−/− (HA-JNK2) cells were stronger compared with those in JNK2−/− (Vector).

Next, real-time PCR was conducted to compare hif-1α mRNA content. It was found that the JNK2 deficiency caused reduction in hif-1α mRNA expression in both knock-out and knockdown systems (Fig. 3D). Therefore, hif-1α promoter-driven luciferase reporter (38, 48) was employed to compare hif-1α transcription among those three transfectants. As shown in Fig. 3E, knock-out of JNK2 increased hif-1α transcription. The higher transcription of hif-1α observed in JNK2−/− cells might result from an adaptive strategy developed by the cells to compensate for the impaired hif-1α mRNA expression. Then hif-1α mRNA stabilities were detected in WT (Vector), JNK2−/− (Vector), and JNK2−/− (HA-JNK2) cells using actinomycin D (Act D) to inhibit mRNA transcription. The half-life of hif-1α mRNA (T1/2) in WT (Vector) cells and JNK2−/− (HA-JNK2) cells was 8.79 h and 10.63 h, respectively. However, in JNK2−/− (Vector) cells it was reduced to 5.80 h (Fig. 3F). Also, more rapid hif-1α mRNA degradation rate was observed when JNK2 was knocked down in NIH3T3 cells (Fig. 3G). The half-life of hif-1α mRNA was about 9.75 h in non-silencing NIH3T3 cells, but it was reduced to 2.27 h in shRNA JNK2 cells (Fig. 3F).

Another mRNA transcription inhibitor, cordycepin (50), was used to determine mRNA stability. However, in the current studies, the higher transcription of hif-1α was not obviously elevated in JNK2−/− cells, but it was reduced to 2.27 h in shRNA JNK2 cells (Fig. 3F). Therefore, it was anticipated that nucleolin might regulate hif-1α mRNA stability.

To test whether nucleolin could bind to hif-1α mRNA, RNA-IP assay was carried out in which anti-nucleolin antibody was used to pull down all mRNAs that physically interacted with nucleolin protein. Messenger RNA was then extracted from the precipitated complex and reverse transcript-PCR was performed to detect the presence of hif-1α mRNA. As shown in Fig. 4H, hif-1α mRNA was found in the immune-complex pulled down by anti-nucleolin antibody, but not present in the precipitation that was pulled down by control IgG, indicating that nucleolin could interact with hif-1α mRNA.

In addition, the RNA pull down assay was performed to reversely confirm the physical binding of nucleolin to hif-1α mRNA 3′-UTR. Briefly, an in vitro transcribed RNA containing 3′-UTR of hif-1α mRNA (1132 pb, 2994–4125 relative to the transcription starting site of NM_010431.2) was labeled with biotin, and incubated with WCL and captured by magnetic streptavidin beads. The proteins bound to 3′-UTR of hif-1α mRNA were analyzed by Western blotting assay, and nucleolin was found in the magnetic particles-bound complex, indicating the interaction of nucleolin with hif-1α mRNA 3′-UTR (Fig. 4F). HnR, another RNA-binding protein, was used as a negative control to indicate the specific binding of nucleolin to hif-1α mRNA 3′-UTR (Fig. 4F). Next, hif-1α mRNA turnover rates were compared between non-silencing cells and shRNA nucleolin MEFs. As shown in Fig. 4I, knockdown of nucleolin rendered more rapid degradation of hif-1α mRNA (T1/2 = 2.08 h) as compared with that in non-silencing cells (T1/2 = 3.43 h), suggesting that nucleolin was involved in regulating hif-1α mRNA turnover. Collectively, our results demonstrated that nucleolin could bind to and stabilize hif-1α mRNA, by which nucleolin mediated HIF-1α protein induction following nickel treatment.

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Nucleolin has been reported to be involved in JNK-mediated IL-2 mRNA stabilization in activated T-cells (53). To determine whether nucleolin was required for regulation of HIF-1α expression, two sets of shRNAs targeting different sequences of mouse nucleolin mRNA were used to exclude the possibility of off-target effect. As shown in Fig. 4C (upper panel), both sets of shRNAs could efficiently reduce nucleolin expression. More importantly, the induction of HIF-1α protein by nickel treatment was greatly decreased in shRNA nucleolin transfectants at 12 and 24 h compared with that in Non-silencing transfectants (Fig. 4C, lower panel). Similar results were obtained in NIH3T3 cells using two shRNAs of nucleolin (Fig. 4D). Following these, HRE luciferase reporter was transfected into non-silencing and shRNA nucleolin MEFs. It was found that the HRE induction was attenuated in either shRNA nucleolin transfectants following nickel exposure (Fig. 4E). The result from real-time PCR further revealed a decrease of hif-1α mRNA level in shRNA nucleolin transfectants (Fig. 4F). However, the results of hif-1α promoter-driven luciferase assay showed that hif-1α transcription was at comparable levels between non-silencing cells and shRNA-nucleolin cells (Fig. 4G), indicating that nucleolin did not inhibit hif-1α gene transcription. Thus, it was anticipated that nucleolin might regulate hif-1α mRNA stability.
knockdown method led to down-regulation of nucleolin expression. In contrast, JNK1 deficiency did not cause obvious change in nucleolin expression (Fig. 5B). The result from RNA-IP assay showed that binding of nucleolin to hif-1α mRNA was reduced in JNK2−/− (Vector) cells when compared with those in WT(Vector) and JNK2−/− (HA-JNK2) cells (Fig. 5C). A reduction in the mRNA level of nucleolin was detected in JNK2-deficient cells by reverse transcript-PCR (Fig. 5D), while transcription of nucleolin was increased in JNK2−/− cells, as shown in nucleolin-promoter luciferase reporter assay (Fig. 5E). The degradation of nucleolin mRNA was found more rapid in JNK2−/− cells (T1/2 = 0.96 h) compared with that in WT cells (T1/2 = 1.60 h) following Act D treatment (Fig. 5F). This result was reproducible when using shRNA JNK2 cells (Fig. 5G). The half-life of nucleolin mRNA was 3.45 times longer in non-silencing cells (T1/2 = 5.14 h) compared with that in shRNA JNK2 cells (T1/2 = 1.49 h). To confirm whether it was through nucleolin that JNK2 regulated HIF-1α expression, GFP-tagged nucleolin was transfected into JNK2−/− cells. We found that the hif-1α mRNA was elevated via Nucleolin by overexpressing GFP-nucleolin in JNK2−/− cells (Fig. 5H), and the HIF-1α protein induction was also restored after nickel exposure (Fig. 5I). Taken together, it was concluded that JNK2 regulated nucleolin expression by stabilizing its mRNA, which further contributed to HIF-1α induction in nickel response.

**DISCUSSION**

JNKs are members of the superfamily of MAPks (54). These types of enzymes are involved in the regulation of various mammalian physiological events, including cell proliferation, cell death, DNA repair, and metabolism (55). In the study reported here, JNK2 was found to be involved in the regulation of HIF-1α
expression following nickel treatment via regulation of its mRNA stability. Further studies revealed that JNK2 mediated nucleolin protein expression, which could bind to hif-1α mRNA and increase its stability.

There are three genes, i.e. jnk1, jnk2, and jnk3, encoding for respective protein products; alternatively-spliced variants of the three transcripts give rise to four JNK1 isoforms, four JNK2 isoforms, and two JNK3 isoforms (56). Although JNK1 and
JNK2 are presumed to operate in a redundant fashion, it is noteworthy that JNK1 and JNK2 function in opposite manners in a number of experimental systems (58, 59). For example, JNK1, but not JNK2, was found to be responsible for cyclooxygenase-2 induction following nickel exposure (49). Similarly, skin tumor formation induced by the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) was suppressed in JNK2−/− mice (58), but the tumor incidence increased in JNK1−/− mice (59). Recently, JNK1 was found to affect the expression and the function of the Hsp90/70 chaperone proteins, thereby preventing the degradation of HIF-1α protein in a Von Hippel-Lindau (VHL)-independent manner (7). In the study reported here, it was further demonstrated that JNK2 was involved in regulating HIF-1α expression through controlling its mRNA stability, which was different from the molecular mechanism operated by JNK1 (Fig. 5).

In the current studies, it was first noticed that the migrating pattern of HIF-1α protein on an SDS-polyacrylamide gel was different between WT and JNK2−/− cells. Specifically, a selective loss of the slower migrating band of HIF-1α species (presumed phosphorylated) was observed in JNK2−/− cells compared with that in WT cells following nickel treatment. The phenomenon was reproduced under hypoxia and other chemical mimicked hypoxia conditions, and was further confirmed using shRNA knockdown approach and restoration of the exogenous jnk2 gene back into knock-out cells. One potential cause for the loss of the slower-migrating band seen in JNK2−/− cells was due to a defect in total HIF-1α protein synthesis, which may lead to impairment in any types of modification of HIF-1α protein, including phosphorylation. An alternative explanation was based on an equal synthesis of HIF-1α protein in WT and knock-out cells, while less phosphor-groups were conjugated to HIF-1α protein in JNK2-deficient cells. To further explore this question, we removed global phosphor-groups by addition of nonspecific protein phosphatase, λ phosphatase into the whole cell lysate, and found that the amount of HIF-1α proteins synthesized in nickel-treated JNK2−/− cells was attenuated in comparison to that in WT cells, therefore the first hypothesis was supported. This supposition was further confirmed by pulse assay using 35S-labeled methionine and cysteine to monitor the de novo HIF-1α protein synthesis rate.

The result of reverse transcript-PCR showed that the expression level of hif-1α mRNA was down-regulated in JNK2−/− cells in comparison with that in WT cells. Moreover, the results from hif-1α promoter-driven luciferase assay excluded the feasibility regarding the transcriptional regulation of hif-1α expression by JNK2. Instead, a more rapid hif-1α mRNA degradation rate was observed in JNK2−/− cells following treatment with transcription inhibitors, demonstrating that JNK2 up-regulated hif-1α mRNA expression level via increasing its stability.

Nucleolin is an important nucleus protein and functions in fundamental DNA and RNA metabolism (40). Previous work revealed that nucleolin had high affinity to G-quadruplex structure (27), including the sequences in the 5′-UTR region of hif-1α mRNA. The study reported here provided in vivo evidence for the physical interaction between nucleolin and hif-1α mRNA using RNA-IP assay with nucleolin antibody. However, in contrast to the previous report from Gonzalea et al., who showed binding of nucleolin to 5′-UTR of hif-1α in vitro filter binding assay (27), our result demonstrated that the interaction of nucleolin with hif-1α mRNA occurred at 3′-UTR region in intact cells. In addition, this kind of interaction facilitated hif-1α mRNA stability, evident from the results of mRNA decay assay, which showed that nucleolin knockdown shortened hif-1α mRNA half-life.

Nucleolin has been reported to be required for JNK-mediated IL-2 mRNA stabilization in activated T-cells (53). The details of the mechanisms responsible for JNK2 regulation of nucleolin expression are currently under investigation in our laboratory. Reverse transcript-PCR result revealed a reduction in the nucleolin mRNA amount in JNK2-deficient cells. But the nucleolin gene transcription was comparable between WT and knock-out cells, as determined by nucleolin promoter reporter. Similar with what we identified in the case of hif-1α, nucleolin mRNA degradation rate was elevated in JNK2-deficient cells. However at the present stage, we are uncertain whether JNK2 regulated global mRNA stability through a commonly shared mechanism or specifically affected mRNA stability of hif-1α and nucleolin.

Collectively, our present studies demonstrate that JNK2 was involved in regulating HIF-1α expression in nickel response through maintaining its mRNA stability. Nucleolin might be a mediator for JNK2 in controlling hif-1α mRNA decay. Therefore, it was suggested that although JNK1 and JNK2 were both involved in the regulation of HIF-1α expression (7), they performed in different mechanisms, thus providing more evidence for the distinct molecular scenarios of each isoform.

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