Hypoxia and nickel inhibit histone demethylase JMJD1A and repress Spry2 expression in human bronchial epithelial BEAS-2B cells

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Introduction

Epigenetic mechanisms, which include DNA methylation and histone modifications, are ubiquitously involved in regulation of gene expression. Environmental factors can often affect regulatory mechanisms of gene transcription and lead to alterations of gene expression pattern. These gene expression alterations help the organisms adapt to the environment but may also inappropriately contribute to disease development. To date, aberrant epigenetic changes and subsequent gene expression alterations have been implicated in development of many human diseases, such as cancers, cardiovascular diseases, type II diabetes and obesity (1,2). However, little is known about how pathogenic environmental factors contribute to development of these diseases by affecting epigenetic regulatory mechanisms.

Our group and others have recently shown that hypoxia and several environmental carcinogens (e.g. nickel, arsenic and chromium) increase global histone methylations on H3K4, H3K9 and/or H3K36, which is probably mediated by inactivation of histone demethylases (3–5). Two families of histone demethylases, flavin-dependent amine oxidases and Jmjc-domain containing histone demethylases, have been recently discovered. In the latter family of histone demethylase, the Jmjc domain is essential for binding of the cofactors (iron and 2-oxoglutarate) and catalyzing oxidative demethylation on histone lysines (6,7). Because of their common requirement of oxygen for demethylation reaction, these Jmjc-domain-containing demethylases are generally less active under hypoxia (8). In contrast to hypoxia, our recent studies have shown that nickel inactivates these iron- and 2-oxoglutarate-dependent enzymes by replacing the cofactor ion at the iron-binding sites of these enzymes (9,10). However, it is still unclear how inactivation of these histone demethylases may be involved in human diseases, such as cancer development.

In this study, we chose one Jmjc-domain-containing histone demethylase, JMJD1A, to study how its inactivation may affect tumorigenesis. JMJD1A demethylates both di- or mono-methylated histone H3 lysine 9 (H3K9me2 and H3K9me1), but not H3K9me3 (11). Both H3K9me1 and H3K9me2 are well associated with repressed gene promoters (12), although H3K9me2 has also been reported to be dynamically present in the transcribed region of some active genes in mammalian chromatin (13). In agreement with its function as a H3K9 demethylase, JMJD1A acts as a coactivator for androgen receptor to enhance transcription of androgen receptor-targeted genes in prostate cells (11). Several recent studies have also shown that JMJD1A is a positive regulator of genes involved in spermatogenesis, smooth muscle cell differentiation, self-renewal of embryonic stem cells and energy metabolism and weight control, suggesting that this demethylase has multiple functions across various biological processes (14–17).

Here, by using Affymetrix GeneChip and ChIP-on-chip technologies, we identified Spry2 gene, a key regulator of receptor tyrosine kinase/extracellular signal-regulated kinase (ERK) signaling, as one of the JMJD1A-targeted genes in human bronchial epithelial BEAS-2B cells. Both hypoxia and nickel exposure increased the level of H3K9me2 at the Spry2 promoter by inhibiting JMJD1A, which probably led to a decreased expression of Spry2 in BEAS-2B cells. Repression of Spry2 potentiated the nickel-induced ERK phosphorylation, and forced expression of Spry2 in BEAS-2B cells decreased the nickel-induced ERK phosphorylation and significantly suppressed nickel-induced anchorage-independent growth. Taken together, our results suggest that histone demethylases could be targets of environmental carcinogens and their inhibition may lead to altered gene expression and eventually carcinogenesis.

Experimental procedures

Cell culture

Human bronchial epithelial BEAS-2B cells, mouse embryonic fibroblast hypoxia-inducible factor-1 alpha (HIF-1α) proficient (HIF-1α+/+) and deficient (HIF-1α−/−) cells (kindly provided by Dr R.Johnson, University of California at San Diego) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Invitrogen). For hypoxia exposure, cells were incubated in a chamber (Valencia, CA) and Invitrogen, respectively. siRNA were transiently transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen) by following the reverse transfection protocol provided by the manufacturer.

Abbreviations: ChIP, chromatin immunoprecipitation; DSG, disuccinimidyl glutarate; ERK, extracellular signal-regulated kinase; HIF-1α, hypoxia-inducible factor-1 alpha; MEF, mouse embryonic fibroblast; mRNA, messenger RNA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RTK, receptor tyrosine kinase; siRNA, small interfering RNA.

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Plasmids and establishment of stable transfectants
The pcDNA3-Flag-JMJD1A vector and its mutant vector, pcDNA3-Flag-JMJD1A H1120Y, were kindly provided from Dr Y.Zhang (University of North Carolina at Chapel Hill). The pHM6-HA-SPRY2 and its empty vector, pHM6-HA, were kindly provided by Dr T.Patel (Loyola University Chicago). Transfection into BEAS-2B was performed using Lipofectamine LTX reagent (Invitrogen). To select stable transfectants, BEAS-2B cells were selected by G418 (Invitrogen) at 1 mg/ml concentration for 3 weeks after transfection. Individual clones were retrieved and expression of exogenous HA-SPRY2 was examined by western blot.

Western blot
Cells were lysed with RIPA buffer and the lysates collected by scraping. The samples were passed through 21 gauge needles and centrifuged at 10 000 g for 10 min. The supernatants were collected as the whole cell lysates. For some experiments (ERK phosphorylation and HIF-1α), whole cell lysates were collected using lysis buffer A (1% sodium dodecyl sulfate, 1.0 mM sodium orthovanadate and 10 mM Tris; pH 7.4) at boiling temperature as described previously (19). Western blots were carried out as described previously (19).

GeneChip, polymerase chain reaction and quantitative real-time–polymerase chain reaction
Total RNA was extracted from cells using Trizol by following the manufacturer’s protocol. The GeneChip analysis was performed as described previously (20). The data were analyzed using GeneSpring software (Agilent, Santa Clara, CA). To verify gene expression changes found in GeneChip assay, conventional polymerase chain reaction (PCR) was performed. Complementary DNA was synthesized using SuperScript® III First-Strand Synthesis SuperMix Kit (Invitrogen). The primers used for semi-quantitative PCR are as following: Jmjd1a sense 5'-AATCCAGAATGCTCCCATGAG-3' and antisense 5'-CA-CATATCCAAAACC-ACACCG-3' and Spry2 sense 5'-TGGTTCA T-3' and antisense 5'-AGGGCTAT-3'. For quantitative real-time PCR, SYBR Green I-based method was employed using β-actin messenger RNA (mRNA) as the reference gene. The primer sequences for Jmjd1a are: 5'- TCTGTTCACAAAGCTATGATG-3' (sense) and 5'-AGGGCTAT-CATATCCAAAACC-3' (antisense). Each real-time PCR (20 μl) contained 1× real-time PCR SYBR Green master buffer (Applied Biosystems, Carlsbad, CA), 10 nM primers, 0.25–1 μl template complementary DNA. The PCR was completed on ABI Prism 7400 Sequence Detector System (Applied Biosystems) using the following program: 95°C for 10 min, then 40 cycles of 95°C for 20 s and 60°C for 1 min. Relative quantification of different gene expression against β-actin mRNA was calculated using comparative 2-ΔΔCt value.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assay was performed as described previously with some modifications (21). For ChIP with H3K9me2 antibody, cells were cross-linked with 1% formaldehyde (Sigma, St. Louis, MO) at room temperature for 10 min. For ChIP with JMJD1A and HIF antibodies, cells were double cross-linked with disuccinimidyl glutarate (DSG; Pierce, Rockford, IL) and formaldehyde. DSG forms cross-links over 7Å, which helps in the stabilization of large multi-protein complexes. Several independent researchers have successfully used this double cross-linking method to detect the binding of transcriptional co-regulators (e.g. nuclear factor-kappaB) to chromatin, and these bindings were not possible to be detected using conventional formaldehyde cross-link (22,23). Cells were briefly rinsed with phosphate-buffered saline (PBS) and then first cross-linked with 10 ml of 2 mM DSG in PBS per 15 cm dish at room temperature for 45 min. A 0.5 M stock concentration of DSG was prepared immediately before use by dissolving DSG in 100% dimethyl sulfoxide. After DSG cross-linking, cells were then briefly rinsed with PBS and subsequently fixed with 10 ml 1% (vol/vol) formaldehyde (Sigma) in PBS at room temperature for 10 min. Glycine was then added to 125 mM final concentration to neutralize formaldehyde. The cells were rinsed with PBS twice and collected by scraping. ChIP assay was performed with EZ-ChIP kit (Millipore) following the manufacturer’s protocol. Chromatin–protein complexes were immunoprecipitated with 5 μg anti-H3K9me2 (Ab1220 from Abcam (Cambridge, MA) or 17-648 from Millipore), anti-HIF-1α (NB100-105; Novus Biologicals, Littleton, CO), or anti-HIF-1β (SC-5580; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-JMJD1A (A301-538A or A501-250; Bethyl). The primers used for PCR amplification are: Jmd1 promoter sense 5'-TCTGTTCACAAAGCTATGATG-3' and antisense 5'-AGGGCTAT-CATATCCAAAACC-3' and Spry2 promoter sense 5'-CAGGGTAGCT-GCAAA TGGA T-3'.

ChIP-on-chip
To map JMJD1A in gene promoter regions in human genome, ChIP-on-chip assays were performed. The experiment was performed by following the protocol provided by Affymetrix. In brief, about 5 × 10⁶ Beas-2B cells were transfected with scramble siRNA or JMJD1A siRNA. Three days after transfection, cells were double cross-linked with DSG and formaldehyde as described above. After cell collection, cell pellets were washed three times with 10 ml lysis buffer per wash. The pellets were resuspended in the lysis buffer supplemented with 1 mM CaCl₂ and sonicated to achieve an average length of chromatin in a range of 200–1000 bp by using a Diagenode Bioruptor (Diagene, Denville, NJ). The samples were diluted with 5 vol of dilution buffer. Following a preclearing procedure, the samples were subject to immunoprecipitation with 15 μg JMJD1A Ab (A301-539A; Bethyl), and the antibody–chromatin complex was collected using proteinase-sepharose beads (Amersham, Piscataway, NJ). The immunoprecipitated complex was washed, eluted and incubated with proteinase K at 65°C overnight. The immunoprecipitated DNA was purified using Affymetrix cDNA cleanup column and quantified by incubating with the Quanti-IT Picogreen dsDNA reagent (Invitrogen). About 36 ng immunoprecipitated DNA was subjected to random priming (primer A: 5'-GTTTCCAGTCAAGCTTGCT[NC12]G-3') and then 32 cycles of PCR (primer B: 5'-GTTTCCAGTCAAGCTTGCT-3') with a final concentration of 2 mM MgCl₂ in each PCR (Note: no PCR amplification was found at the concentration of MgCl₂ [0.75 mM]). The PCR amplified product was found at the concentration of a DNA of 0.75 mM) recommended by Affymetrix, Santa Clara, CA). Different PCR amplification cycles were tested using Input DNA to ensure that the amplification fell in the linear range of PCR. The amplified DNA was purified and quantified using a NanoDrop, and ~7.5 μg DNA was obtained per 100 μl reaction. The amplified DNA was fragmented and labeled using the DNA Terminal Labeling Kit (Affymetrix) and then hybridized to Affymetrix GeneChip Human Promoter 1.0R Array.

ChIP-on-chip data analysis
Data from the arrays were normalized and analyzed using the CisGenome software (24). The intensities of signals were compared between JMJD1A siRNA versus scramble siRNA samples. The peaks with lower signals in JMJD1A-knockdown samples and their associated closest genes were identified. The overlap of the gene lists between the ChIP-on-chip and expression arrays was analyzed using a Venn diagram program (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Soft agar colony formation assay
Five thousand BEAS-2B cells or their derivatives were grown on a 35 mm plate containing 0.5% base agar and 0.34% top agar in Dulbecco’s modified Eagle’s medium complete medium and incubated at 37°C for 3–4 weeks. Plates were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Sciences, Indianapolis, IN) overnight. Colonies were counted using Kodak 1D program.
Statistical analysis
The two-tailed Student’s t-test was used to determine the significance of differences between the treated samples and controls. The difference was considered significant at $P < 0.05$.

Results
Identification of JMJD1A target genes in human bronchial epithelial BEAS-2B cells
To determine whether JMJD1A is involved in regulation of gene expression in human bronchial epithelial BEAS-2B cells, we used siRNA to knockdown JMJD1A and then used Affymetrix GeneChip microarrays to analyze genome-wide gene expression changes. As shown in Figure 1A, JMJD1A expression was dramatically decreased after transient transfection with specific siRNA. We analyzed the genome-wide gene expression profile after knockdown of JMJD1A by using a 2-fold change as a cutoff. In total, 2590 genes were found to have >2-fold decrease in expression, whereas an almost identical number of genes (2216 genes) had >2 fold increase of gene expression. These changes in gene expressions are probably caused directly or indirectly (e.g. mediated through other transcriptional factors) by loss of JMJD1A.

To identify which genes were directly regulated by JMJD1A, we performed ChIP-on-chip assay to map JMJD1A in gene promoter regions by using Affymetrix GeneChip® Human Promoter 1.0R Array, which contains the region spanning ∼7.5 kb upstream through 2.45 kb downstream of 5′ transcription start sites of 25 500 human genes. The experiment was performed in BEAS-2B cells transfected with either JMJD1A siRNA or scramble siRNA. A total of 629 regions that were associated with 609 closest genes were identified with either JMJD1A siRNA or scramble siRNA. The experiment was performed in BEAS-2B cells transfected with siRNA against JMJD1A. Three days after transfection, whole cell lysates were collected and the expression of endogenous JMJD1A was detected by immunoblotting. (Figure 2C). Venn diagram analysis of the potential JMJD1A target genes. The genes in the yellow circle were found to have JMJD1A-binding peaks close to their gene loci as identified by ChIP-on-chip assay, whereas the genes in the green circle showed >2-fold decrease in gene expression after knockdown of JMJD1A in BEAS-2B cells. (C) Distribution of manually curated gene ontology annotations for potential JMJD1A targets. In some instances, a gene is assigned to more than one category. The percentage refers to the number of genes within any particular gene ontology category in relation to the total number of JMJD1A-targeted genes ($N = 68$).

JMJD1A regulates Spry2 gene expression in BEAS-2B cells
We next chose Spry2, a key gene in regulation of receptor tyrosine kinase (RTK)–ERK signaling pathway (25), from the category ‘signaling transductions’ for further study. We first verified the decrease of its gene expression after knockdown of JMJD1A by using semi-quantitative PCR (Figure 2A). The SPRY2 protein level was also found to be lower in the JMJD1A-knockdown cells (Figure 2B). To exclude the possibility that decrease of Spry2 expression is due to off-target effect of JMJD1A siRNA knockdown, another siRNA targeting a different sequence was used to knock down JMJD1A in BEAS-2B cells. A similar decrease in SPRY2 protein level was observed in the two samples with JMJD1A knockdown by different siRNAs, indicating that this decrease of SPRY2 expression was caused by elimination of JMJD1A and not due to off-target effects of siRNA (Figure 2C). Consistent with above findings, overexpression of JMJD1A WT led to a much higher increase of Spry2 mRNA level when compared with overexpression of its mutant form (JMJD1A H1120Y), which has mutation in the iron-binding motif and is not enzymatically active (Figure 2D). Similarly, SPRY2 protein level was also found to be elevated when JMJD1A was overexpressed (Figure 2E). Taken together, these results demonstrated that JMJD1A is involved in regulation of Spry2 expression.
Hypoxia or nickel exposure decreased expression of Spry2

Because JMJD1A requires several cofactors (e.g., oxygen and iron) to be active, its demethylase activity is inhibited under condition of low oxygen. Consistent with the findings of JMJD1A knockdown, hypoxia decreased both gene expression and protein levels of Spry2 (Figure 3A–B). We have previously reported that nickel inactivated JMJD1A by interfering with binding of ferrous iron to this demethylase (9,10). Results from Figure 3C–D showed that nickel exposure at the 100 μM concentration decreased both gene expression and protein levels of Spry2. Interestingly, both mRNA and protein levels of JMJD1A were found to be upregulated in the BEAS-2B cells that were exposed to hypoxia and nickel (Figure 3B–D), suggesting that both hypoxia and nickel exposure decreased Spry2 expression through affecting JMJD1A.

The demethylase activity of JMJD1A is essential for its direct regulation of Spry2 expression

To verify the findings of ChIP-on-chip, we examined the association of JMJD1A with the Spry2 promoter using ChIP assays. The results in Figure 4A demonstrated that JMJD1A bound to the Spry2 promoter in BEAS-2B cells, and the signal for this binding was greatly weakened when JMJD1A was knocked down. In support that JMJD1A functions as a histone demethylase at the Spry2 promoter, knockdown of JMJD1A significantly increased H3K9me2 level at the Spry2 promoter (Figure 4A). Having established that JMJD1A functions as a histone demethylase at the Spry2 promoter, we next analyzed the changes of JMJD1A binding and H3K9me2 level in the hypoxia-exposed cells. In agreement with the finding that hypoxia-exposed BEAS-2B cells have a higher expression of JMJD1A (Figure 3B), more JMJD1A was found to be associated with the Spry2 promoter in these cells (Figure 4B). Although there was more JMJD1A associated with the Spry2 promoter, the H3K9me2 level was found to be higher at the same genomic site in the hypoxia-treated cells, which is comparable with the elevation observed in the JMJD1A-knockdown cells (Figure 4C). These results suggest that JMJD1A associated with Spry2 promoter was less active under hypoxia. Similar findings were also observed in cells exposed to nickel. As shown in Figure 4D, there was more JMJD1A and H3K9me2 associated with Spry2 promoter in the nickel-exposed BEAS-2B cells. Taken together, these results demonstrated that JMJD1A functions as a histone H3K9 demethylase at the Spry2 promoter and its demethylase activity was inhibited under hypoxia and nickel exposures.

Hypoxia and nickel ions induce JMJD1A expression through HIF1-α

Several groups have recently reported that hypoxia increases the expression of JMJD1A through HIF-1α (8,26,27), but it is still unclear how nickel upregulates JMJD1A expression. Under hypoxia condition, HIF-1α is stabilized and bound to HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator) to form a heterodimeric transcriptional factor, HIF-1. HIF-1 binds to DNA at hypoxia response element and upregulates the expression of genes that function to increase oxygen availability or to adjust intracellular metabolism to oxygen deprivation, resulting in an improved cell survival under hypoxia. We have previously reported that nickel also activates HIF signaling pathway by inhibiting HIF-prolyl hydroxylases that promote the degradation of HIF-1α (19). To examine whether HIF-1 is involved in the hypoxia- and nickel-induced upregulation of JMjd1a, we measured Jmdla expression in HIF1α−/− mouse embryonic fibroblast (MEF) cells after exposure to nickel ions or hypoxia. Figure 5A shows that both nickel ion and hypoxia exposures increased Jmdla expression in HIF1α−/− MEff cells but not in HIF1α+/− cells, indicating that nickel ions and hypoxia increase Jmdla gene expression through HIF-1α. Next, we examined whether HIF-1α was involved in the nickel-induced upregulation of JMJD1A in BEAS-2B cells. In agreement with the above findings using MEF cells (Figure 5A), the increase of JMJD1A protein by acute nickel ion exposure was
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Fig. 3. Hypoxia and nickel exposure decreased Spry2 expression. (A) Hypoxia decreases Spry2 gene expression in BEAS-2B cells. BEAS-2B cells were exposed to hypoxia (1% O2) for 3 days. Expression of Spry2 was examined using semiquantitative PCR. (B) Hypoxia decreases SPRY2 but increases JMJD1A expression in BEAS-2B cells. BEAS-2B cells were transiently transfected with scramble siRNA or JMJD1A siRNA. One day after transfection, cells were replenished with fresh media and incubated in chambers with either 21 or 1% O2 for 3 days. Expressions of SPRY2 and JMJD1A in these cells were analyzed with immunoblotting. (C) Nickel decreases Spry2 gene expression in BEAS-2B cells. BEAS-2B cells were exposed to 50 or 100 μM NiCl2 for 8 weeks and expression of Spry2 was examined using semiquantitative PCR. (D) Nickel decreases SPRY2 but increases JMJD1A expression in BEAS-2B cells. The expression of SPRY2 and JMJD1A was analyzed by western blot in the cells treated with 100 μM NiCl2 for 8 weeks.

Repression of Spry2 expression potentiates nickel-induced ERK phosphorylation

Given that the expression of Spry2 gene is often decreased in human lung cancers (28), we hypothesize that its downregulation may play a role in carcinogenesis. Because Spry2 is a key regulator of RTK-ERK signaling pathway, we examined whether a decreased expression of SPRY2 could lead to ERK activation under hypoxia or nickel exposure. We measured ERK phosphorylation in the nickel-exposed BEAS-2B cells and found that ERK phosphorylation was significantly increased compared with untreated cells (Figure 6A). By contrast, no increase of ERK phosphorylation was observed in hypoxia-exposed cells (data not shown), suggesting that decrease of SPRY2 alone is not sufficient to activate ERK signaling pathway. To further understand the roles of JMJD1A and SPRY2 in the nickel-induced ERK signaling, we measured ERK phosphorylation in the nickel-exposed BEAS-2B cells after either knockdown or overexpression of SPRY2 or JMJD1A. Although knockdown of SPRY2 or JMJD1A was not found to affect the basal level of ERK phosphorylation in BEAS-2B cells, these treatments enhanced the increases in ERK phosphorylation in the nickel-exposed BEAS-2B cells (Figure 6C). Taken together, these results demonstrate that JMJD1A negatively regulates the nickel ion-induced ERK signaling through SPRY2.

Repression of Spry2 expression potentiates nickel-induced anchorage-independent growth

Activation of ERK signaling pathway is an important event in tumorigenesis. ERK signaling has been implicated in cell migration, regulation of apoptosis, angiogenesis and other events that are essential for tumorigenesis and tumor metastasis (29). We chose to use anchorage-independent growth, a hallmark of cell transformation, as indicative of progression from a non-tumorigenic state to a tumorigenic state after nickel treatment. Consistent with our previous findings that long-term nickel treatment led to cell transformation in several mammalian cell lines (20), continuous exposure of BEAS-2B cells to nickel ions dramatically increased their anchorage-independent growth potential (Figure 7A).

To address whether Spry2 repression played any role in the nickel-induced cell transformation, we generated the derivatives of BEAS-2B cells (BEAS-2B-HA-SPRY2) that stably express exogenous HA-SPRY2 at a level of expression similar to the endogenous gene
As previously reported by several groups (30), the HA-SPRY2 vector expresses two bands. As shown in Figure 7B, one band of HA-SPRY2 was at a similar molecular weight as endogenous SPRY2, whereas the other band was at a lower molecular weight probably because HA tag partially affects SPRY2 phosphorylation. Following exposure to 100 μM nickel ions for 3 weeks, BEAS-2B-HA-SPRY2 cells retained a higher level of total SPRY2 expression (exogenous + endogenous) as compared with the cells stably transfected with HA empty vector (BEAS-2B-HA) (Figure 7B). We next examined the anchorage-independent growth potential of the nickel-treated BEAS-2B-HA-SPRY2 cells. As shown in Figure 7C, BEAS-2B-HA-SPRY2 cells formed a 5-fold lower number of colonies in soft agar as compared with BEAS-2B-HA cells, and a 3 weeks nickel exposure caused a much higher increase of anchorage-independent growth in the BEAS-2B-HA cells as compared with the BEAS-2B-HA-SPRY2 cells. Taken together, these results demonstrated that SPRY2 repression is an essential factor in the nickel-induced cell transformation.

Fig. 5. Nickel ions induce JMJD1A expression through HIF1α. (A) Nickel ion exposure and hypoxia fail to induce JMJD1a in HIF1α-deficient MEF cells. HIF1α-proficient (HIF1α+/+) and -deficient (HIF1α−/−) MEF cells were exposed to 250 μM NiCl2 or hypoxia (1% O2) for 24 h. Expression of JMJD1a was then assessed by immunoblotting. (B) Knockdown of HIF1α blocks the induction of JMJD1A by nickel ions in BEAS-2B cells. Cells were transfected with siRNA against HIF1α and were then exposed to 300 μM NiCl2 for 24 h. Expression of JMJD1A was then assessed by immunoblotting. (C) Knockdown of HIF1α blocks the induction of Jmjd1a mRNA by nickel ions in BEAS-2B cells. Cells were treated under the same condition as described in (B). The relative amount of Jmjd1a mRNA was measured by quantitative real-time PCR and normalized to that of β-actin. Values are means ± SD for triplicates. (D) Binding of HIF-1α to the promoter of Jmjd1a gene. BEAS-2B cells were treated with 300 μM NiCl2 for 24 h. ChIP assay was performed to analyze the binding of HIF-1α and HIF-1β (aryl hydrocarbon receptor nuclear translocator) at the hypoxia response element site in the Jmjd1a promoter.

Fig. 6. Repression of Spry2 expression potentiates nickel-induced ERK phosphorylation. (A) ERK activation in the BEAS-2B cells treated with 100 μM NiCl2 for 8 weeks. Whole cell extracts were prepared from BEAS-2B and nickel-treated cells, and phosphorylation levels of ERK1/2 at Thr202/Tyr204 and total ERK1/2 were assessed by immunoblotting. (B) Knockdown of SPRY2 or JMJD1A potentiates nickel-induced ERK phosphorylation. Control and nickel-treated BEAS-2B cells were transfected with siRNA against SPRY2 or JMJD1A. The phosphorylation forms and total ERK1/2 were then assessed by immunoblotting. (C) Overexpression of HA-SPRY2 or JMJD1A attenuates ERK phosphorylation in the nickel-treated BEAS-2B cells. Control and nickel-treated BEAS-2B cells were transfected with HA-SPRY2 or Flag-JMJD1A expression vector. The phosphorylation forms and total ERK1/2 were then assessed by immunoblotting.
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Fig. 7. Repression of Spry2 expression potentiates nickel-induced anchorage-independent growth in BEAS-2B cells. (A) Nickel-treated BEAS-2B cells (100 μM NiCl2 for 8 weeks) have increased anchorage-independent growth. 6 x 10^3 control and nickel-treated BEAS-2B cells were seeded in soft agar. After 4 weeks incubation, colonies grown in soft agar were stained, photographed and counted. Values are presented as mean ± SD, N = 3. (B) Attenuation of the nickel ion-induced ERK phosphorylation in BEAS-2B cells that stably express exogenous HA-SPRY2. BEAS-2B cells that stably express HA or HA-SPRY2 were treated with 100 μM NiCl2 for 3 weeks. Immunoblotting was utilized to assess the expression of endogenous SPRY2, exogenous HA-SPRY2, as well as the phosphorylation forms and total ERK1/2. The HA-SPRY2 vector expresses two bands, a phenomenon reported previously by several groups (30). Western blot band intensities were quantified using an ImageJ program, and results were expressed as top band/bottom band. (C) Stable expression of SPRY2 inhibits nickel-induced anchorage-independent growth in BEAS-2B cells. BEAS-2B cells that stably express HA or HA-SPRY2 were treated with 100 μM NiCl2 for 3 weeks and then seeded in soft agar. After 4 weeks, colonies were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride solution, photographed and counted. Values are presented as mean ± SD, N = 3.

Discussion

The results of this study support the notion that inactivation of histone demethylase(s) may contribute to tumorigenesis. We found that expression of Spry2 gene was downregulated by hypoxia or nickel through inhibition of JMJD1A. Decreased expression of Spry2 potentiated nickel-induced ERK activation and was essential for development of anchorage-independent growth in the nickel-treated cells.

Nickel compounds have been well established as human respiratory carcinogens, causing a very high incidence of lung and nasal cancers in nickel refinery workers (31). Injection of nickel compounds into experimental animals induced formation of malignant fibrous histiocytomas and sarcomas, with the p16 and Fhit genes often found to be epigenetically silenced in these cancers (32,33). By using a transgenic cell model with the transgene placed near heterochromatin, we have previously demonstrated that nickel exposure caused a very high frequency of transgene silencing, and levels of DNA methylation and repressive histone marks were significantly elevated at the promoter of the silenced transgene (21,34–36). The results from this study extend our previous finding and demonstrated that nickel exposure also represses endogenous gene expression by inhibiting histone demethylase(s). JMJD1A may also regulate Spry2 through a mechanism unrelated to its demethylation activity since overexpression of mutant JMJD1A vectors appeared to slightly increase the Spry2 mRNA level (Figure 2D).

Recently, several groups have reported that hypoxia induces JMJD1A expression through HIF-1, which binds to the hypoxia response element present at the JMJD1A promoter to enhance transcription (8,26,27). Similar to the findings in these studies, we also observed that nickel ions induce JMJD1A expression through HIF-1α, but not HIF-2α. Besides JmjD1A, three other α-ketoglutarate-dependent histone demethylase genes (Jarid1b, Jmjd2b and Jmjd2c) were found to be upregulated under hypoxia through HIF-1α (3,27). The significance of these genes' upregulation under hypoxia is still not clearly understood. A previous study reported that hypoxia at 1% oxygen repressed the activity of endogenous FIH (factor inhibiting HIF-1; Jmjc-domain containing HIF asparagine hydroxylase), but a more severe hypoxia (0.2% O2) was needed to inhibit exogenously overexpressed FIH (37). Based on this finding, it has been speculated that induction of these histone demethylase genes may compensate for loss of their enzymatic activities under hypoxia to certain extent (26). Although one study reported that blocking HIF signaling by knockdown of HIF-1α increased global H3K4me3 levels in hypoxic Hep3G cells (3), it has also been reported that knockdown of JMJD1A and JMJD2B did not increase global H3K9 methylation levels in hypoxic HeLa cells (8). Our results indicate that the level of JMJD1A induction is, at least, not sufficient to reverse inhibition of Spry2 gene expression caused by hypoxia (1% O2) and nickel.

SPRY2 is a key regulator of the RTK–ERK signaling pathway, with little or no activity toward either c-jun N-terminal kinase or p38 signaling pathway (38). SPRY2 has been shown to negatively regulate the RTK–mitogen-activated protein kinase signaling activated by fibroblast growth factor, platelet-derived growth factor and vascular endothelial growth factor (38–40), but it may also potentiate epidermal growth factor receptor (EGFR) signaling by specifically intercepting c-Cbl-mediated EGFR ubiquitination and subsequent degradation (30,41,42). It has been reported that Src kinase, a receptor tyrosine kinase, was activated in BEAS-2B cells exposed to nickel and its activation was involved in the nickel-induced ERK activation (43). Although many studies reported that hypoxia activated ERK signaling in a variety of cell types (e.g. cardiomyocyte (44) and microvascular...
endothelial cells (45)], there are also some studies showing that hypoxia did not increase ERK phosphorylation in renal epithelial cells (46). In RN46A neuronal cells, it was found that hypoxia increased ERK phosphorylation only at 1 h post-exposure but not at later time intervals (47). Here, we found that hypoxia (1% O2) exposure for 24 h did not increase ERK phosphorylation in BEAS-2B cells, which could be due to a difference in cell type or hypoxia exposure conditions (e.g. O2 concentration and exposure time). Based on the results from this study, downregulation of Spry2 by nickel apparently enhanced the RTK–ERK signaling and restoration of Spry2 expression decreased ERK activation and anchorage-independent growth in nickel-exposed cells. These results are in agreement with previous reports that overexpression of Spry2 inhibited lung tumorigenesis induced by either K-Ras hyperactivation or urethane exposure through antagonizing RTK–ERK signaling (48,49). It has been reported that SPRY2 and pHM6-HA expression vectors.

viding pCDNA-3-Flag-JMJD1A WT and mutant (H1120Y) expression vectors.

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References

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Supplementary material

Supplementary Tables 1–3 can be found at http://carcin.oxfordjournals.org/.

JMJDA inhibition causes Spry2 repression

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