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Nickel exposure induces persistent mesenchymal phenotype in human lung epithelial cells through epigenetic activation of ZEB1

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Abstract

Nickel (Ni) is an environmental and occupational carcinogen, and exposure to Ni is associated with lung and nasal cancers in humans. Furthermore, Ni exposure is implicated in several lung diseases including chronic inflammatory airway diseases, asthma and fibrosis. However, the mutagenic potential of Ni is low and does not correlate with its potent toxicity and carcinogenicity. Therefore, mechanisms underlying Ni exposure-associated diseases remain poorly understood. Since the health risks of environmental exposures often continue post exposure, understanding the exposure effects that persist after the termination of exposure could provide mechanistic insights into diseases. By examining the persistent effects of Ni exposure, we report that Ni induces epithelial-mesenchymal transition (EMT) and that the mesenchymal phenotype remains irreversible even after the termination of exposure. Ni-induced EMT was dependent on the irreversible upregulation of ZEB1, an EMT master regulator, via resolution of its promoter bivalency. ZEB1, upon activation, downregulated its repressors as well as the cell-cell adhesion molecule, E-cadherin, resulting in the cells undergoing EMT and switching to persistent mesenchymal status. ZEB1 depletion in cells exposed to Ni attenuated Ni-induced EMT. Moreover, Ni exposure did not induce EMT in ZEB1-depleted cells. Activation of EMT, during which the epithelial cells lose cell-cell adhesion and become migratory and invasive, plays a major role in asthma, fibrosis, and cancer and metastasis, lung diseases associated with Ni exposure. Therefore, our finding of irreversible epigenetic activation of ZEB1 by Ni exposure and the acquisition of persistent mesenchymal phenotype would have important implications in understanding Ni-induced diseases.

Keywords

Epithelial-mesenchymal transition; nickel; ZEB1; epigenetics; bivalent chromatin

1 INTRODUCTION

Nickel compounds are environmental and occupational pollutants prevalent in the atmosphere due to their widespread use in several industrial processes, as well as extensive consumption of Ni containing products such as stainless steel, batteries, medical devices, coins, jewelry, electric equipment and medical implants [1–4]. In addition, combustion of fossil fuels is a major source of Ni contamination in the atmosphere [5]. Humans can be exposed to Ni through inhalation, ingestion and skin contact [1,2].

Exposure to Ni is associated with a multitude of human health risks, including allergic contact dermatitis, asthma, inflammation, bronchitis, pulmonary fibrosis, pulmonary edema as well as diseases of the kidney and cardiovascular system [6–8]. Moreover, epidemiological studies indicate development of cancer as the most serious outcome of Ni exposure [4,6,7,9–14]. Both water-soluble and -insoluble Ni compounds are carcinogenic [15,16], and animal studies have shown that Ni compounds can induce tumors at the sites of exposure [17]. Therefore, Ni has been classified as a Group 1 Carcinogen by the International Agency for Research on Cancer (IARC) [4,18]. Although Ni is carcinogenic, its mutagenicity is low [4,7,19]. Moreover, Ni does not form DNA adducts [19–21] and the level of oxidative stress induced by Ni is lower than other metals [22]. Therefore, mechanisms underlying the etiology of diseases associated with Ni exposure remain poorly understood.

A number of recent studies suggest that stable, long-term changes to gene expression and cellular regulation caused by environmental exposures likely play a role in disease development. For instance, in smokers, differentially expressed oncogenes, tumor suppressor genes and miRNAs failed to revert to never-smoker levels even years after cessation of smoking [23–25], which likely explains the increased lung cancer risk in former smokers [23]. Similarly, early-life exposure to pesticides and heavy metals is implicated in increased disease susceptibility later in life [26,27]. Therefore, understanding the effects of environmental exposures that persist even after the cessation of exposure could provide mechanistic insights into disease processes.

In this study, we sought to understand the potential mechanisms underlying Ni-induced diseases, by examining the changes to the transcriptional program and cellular regulation that persist after the termination of exposure. We found that Ni exposure-induced transcriptional changes persist long after the termination of exposure. Ni exposure caused epithelial-mesenchymal transition (EMT), and the mesenchymal phenotype persisted even after the cessation of exposure. Our results suggest that irreversible epigenetic activation of ZEB1, an EMT master regulator, is central for the induction of persistent mesenchymal phenotype in the Ni-exposed cells. Activation of EMT, during which the epithelial cells lose cell-cell adhesion and become migratory and invasive, plays a major role in disease processes such as chronic inflammatory airway diseases, asthma, fibrosis, cancer and metastasis, diseases commonly associated with Ni exposure [1,2,6,12,28,29]. Therefore, our studies contribute to the understanding of diseases associated with Ni exposure.

2 MATERIALS AND METHODS

2.1 Cell culture and treatments

Human lung epithelial BEAS-2B cells (ATCC, CRL-9609) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro) supplemented with 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS, Atlanta Biologicals) at 37°C and 5 % CO₂. Human urinary bladder epithelial RT4 cells, a generous gift of Dr. Tang (New York University School of Medicine, NY), were cultured in McCoy's 5A Modified Medium (Life Technologies), supplemented with 10% FBS (Atlanta Biologicals) and 0.5% Penicillin-Streptomycin at 37°C and 5% CO₂. For chronic Ni exposure, 100 µM (50 and 10 µM in Figure 2G) NiCl₂ (N6136, Sigma) was added to the media, and the cells were cultured for 6 weeks (2 weeks in Figure 4B). Following exposure, the cells were washed and cultured in Ni-free medium for 2 weeks to obtain the Ni-washed-out cells. For acute Ni exposure, the cells were exposed to 500 µM NiCl₂ for 72 h. Following exposure, the cells were washed and cultured in Ni-free medium for 2 weeks to obtain the acute Ni-washed-out cells.

For hypoxia cultures, the cell culture medium was pre-equilibrated at 3% O₂ overnight. Untreated BEAS-2B cells growing at 21% O₂ for several generations were transferred to the pre-equilibrated culture medium and incubated at 3% O₂ for 2 weeks in a Panasonic MCO-5M-PA O₂/CO₂ incubator equipped with zirconia sensor and automatic O₂ cylinder switch over system for constant monitoring and maintenance of O₂ levels. CO₂ levels were maintained at 5%. After 2 weeks, one half of the cells were harvested for analysis. The other half of the cells were reverted to normoxia (21% O₂) and cultured for 2 more weeks and then harvested for analysis.

2.2 Isolation of homogenous populations of Ni-exposed cells (Ni-clones)

BEAS-2B cells exposed to 100 µM NiCl₂ for 6 weeks were plated in 15 cm plates at the rate of 1000, 500, 100 and 50 cells per plate in Ni-free medium. Six, well-separated single colonies were randomly isolated and the populations were expanded (Ni-C1 to Ni-C6) in Ni-free medium.

2.3 Western blotting

Total proteins were isolated using Laemmli buffer (Bio-Rad) or RIPA buffer. The proteins separated on SDS-PAGE gels were transferred to nitrocellulose membranes (Bio-Rad) and probed with antibodies against ZEB1 (3396, Cell Signaling), SNAIL (3879, Cell Signaling), TWIST1 (ab50887, Abcam), CDH1 (NBP2-19051, Novus Biologicals) CLDN1 (Cell Signaling, 13255), FN1 (LF-MA0151, Thermo Scientific) and HIF1α (610959, BD Biosciences). Loading control: β actin (ab3280, Abcam).

2.4 RNA isolation and qPCR analysis

Total RNA was isolated using RNeasy kit (Qiagen) according to manufacturer's instructions. cDNA synthesis for protein coding genes and miRNAs was carried out using Superscript III Reverse Transcriptase (Invitrogen) and qScript microRNA cDNA Synthesis Kit (Quantabio, Beverly, MA), respectively. Real time quantitative PCR (qPCR) was performed using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) or FastStart

Universal SYBR Green Master Mix (Roche Diagnostics) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). Statistical significance of qPCR results was evaluated using t-test. *GAPDH* and *SNORD44*: internal controls for protein coding and miRNA genes, respectively.

2.5 RNA-Seq and data analysis

RNA-Seq was performed using RNA samples isolated from two biological replicates. RNA-Seq libraries were prepared using Illumina TruSeq RNA Sample Preparation Kit (RS-122-2002), according to manufacturer's protocol. Data analysis was performed using Wardrobe experimental management system [30], as explained earlier [31,32]. Briefly, gene expression levels were quantified as reads per kilobase of exon per million fragments mapped (RPKM). Genes with RPKM ≥ 1 in at least one experimental condition were considered as expressed. Differential gene expression was calculated using DESeq2 [33] and genes that show ≥ 2.0 fold up- or down-regulation, along with adjusted p-value < 0.01 were considered as differentially expressed. Unsupervised hierarchical clustering analysis of Ni-exposed, Ni-washed-out and time-matched-controls were performed with cluster 3.0 [34] using Euclidean distance. The resulting distance matrix was visualized using Java Treeview 3.0 [35]. Principle component analysis (PCA) was carried out using ClustVis [36]. RNA-Seq data was submitted to Gene Expression Omnibus (GEO) under the accession number GSE95180.

2.6 Canonical pathway analysis

Canonical pathway analysis was done using IPA tool [37]. Enrichment scores (Fisher's exact test, $p < 0.05$) along with the z-scores were used for ranking the top canonical pathways. Heatmaps were generated using R package and Cluster 3.0 [34].

2.7 Chromatin immunoprecipitation (ChIP) analysis

For ChIP, the cells were crosslinked with 1% formaldehyde for 10 minutes at 25°C, and sonicated to obtain 200–500 bp fragments. ChIP was performed as described earlier [31,38], using ChIP-grade antibodies against H3K4me3 (Millipore, 07-473) and H3K27me3 (Millipore, 07-449).

2.8 ZEB1 Knockdown

For ZEB1 shRNA production, the following oligonucleotides were synthesized: 5'-GATCCGTCTGGGTGTAATCGTAAATTCTTCCTGTCAGAAATTTACGATTACACCCA GACTTTTTTG-3'; 5'-AATTCAAAAAGTCTGGGTGTAATCGTAAATTTCTGACAGGAAGAATTTACGATTAC ACCCAGACG-3'. The annealed oligonucleotides were ligated into BamH1/EcoR1 site of pGreenPuro shRNA cloning and expression lentivector (System Biosciences). As control (shControl), a sequence that does not target any known mammalian gene (System Biosciences) was used. Viral packaging was carried out as described earlier [38], by transfecting the shRNA constructs into 293T cells along with viral packaging plasmids pLP/VSVG (Invitrogen) and psPAX2 (Addgene) using Lipofectamine 2000 (Invitrogen). After 48 hours, the viral supernatant was collected and added to BEAS-2B cells in culture. The

infected cells were selected with 0.3 µg/µl puromycin (Sigma) for 15 days. To screen for stable ZEB1 knockdown, the cells were cultured for 30 more days without puromycin selection. Knockdown efficiency was examined at different time points by assessing ZEB1 mRNA (qPCR) and protein levels (western blotting).

2.9 Invasion Assay

Transwell invasion assay was performed using Corning BioCoat Matrigel Invasion Chamber (354481) according to manufacturer's instructions. Briefly, 250,000 cells in serum free DMEM (BEAS-2B cells) or McCoy's 5A Modified Medium (RT4 cells) were seeded in duplicates on matrigel-coated inserts. DMEM supplemented with 10% FBS was added to the lower chamber as a chemo attractant and incubated at 37°C. After 24 h, the non-invading cells on the upper surface were removed by scrubbing. Cells that had migrated to the bottom surface (invasive cells) were then fixed in 100% methanol and stained with 0.5% (w/v) crystal violet and photographed using an inverted microscope at four fields per well and quantified by manual counting. Each assay was performed independently at least three times.

2.10 Wound healing assay

Cells were cultured to 90% confluence in 6-well plates in duplicates. The monolayer was then scratched using a sterile 200 µl pipette tip and the cells were incubated for 24 h in DMEM supplemented with 10% FBS. The scratch was photographed at 0 h and 24 h time points. For quantification, image analysis was performed using Image J software. Each assay was performed independently at least three times.

2.11 Cytotoxicity assay

To examine cytotoxicity, 5000 cells were seeded in triplicates in 96-well plates and incubated at 37°C for 24 h. The cells were then treated with 0–1000 µM NiCl₂ for 72 h. Cytotoxicity was measured at 24, 48 and 72 h time points using Promega CellTiter 96 non-radioactive cell proliferation assay kit (G4001), according to manufacturer's instructions. Statistical significance was evaluated using t-test.

3 RESULTS

3.1 Exposure to nickel causes persistent gene expression changes

Inhalation is the main route of Ni exposure [2]. Consequently, lung is the primary target of Ni exposure, with respiratory and nasal cancers being major outcomes [39]. Therefore, in this study we used immortalized, non-malignant human bronchial epithelial BEAS-2B cells to examine the persistent effects of Ni exposure. Only physiologically relevant doses of Ni, based on previous human studies were used [40]. BEAS-2B cells were exposed to a non-cytotoxic concentration of 100 µM NiCl₂ for 6 weeks (Ni-exposed) (Supplementary Figure S1 and S2). Following exposure, the cells were washed and cultured in NiCl₂-free medium for 2 weeks (Ni-washed-out) (Supplementary Figure S2). Gene expression analysis of Ni-exposed and Ni-washed-out cells was carried out using RNA-Seq (Figure 1A). We found that several genes were differentially expressed (≥ 2 fold up- or down-regulated) only in the presence of Ni, with the expression reverting to basal levels in Ni-washed-out cells

(transiently differentially expressed). However, a subset of genes remained differentially expressed even after the cessation of Ni exposure (persistently differentially expressed). Unsupervised hierarchical clustering analysis showed grouping of the differentially expressed genes into four clusters: i) transiently upregulated, ii) persistently upregulated, iii) transiently downregulated and iv) persistently downregulated (Figure 1A). Moreover, the Ni-exposed and Ni-washed-out samples clustered together signifying shared gene expression profiles (Figure 1A), further suggesting persistence of transcriptional changes caused by Ni exposure.

To gain insight into the biological processes that are potentially impacted by Ni exposure, we next performed gene list enrichment analysis for the identification of over represented canonical pathways. Our results show epithelial-mesenchymal transition (EMT) as the most enriched biological pathway in the persistently differentially expressed genes (Figure 1B). In addition, other cancer related biological processes including Wnt/ β catenin signaling and cancer invasion and metastasis signaling were significantly enriched (Figure 1B). In contrast, the transiently differentially expressed genes did not show a strong association with cancer or metastasis related processes (Figure 1C).

We next considered the possibility of Ni exposure impacting individual cells to different levels due to cell-to-cell variability at a population level, which could result in a heterogeneous population of cells with varying transcriptional profiles. This could potentially introduce selection bias post Ni exposure, since the sub-population of cells that have acquired growth advantage could outcompete the other cells simply by growing better over time. Therefore, we wanted to examine several individual cells exposed to Ni. To accomplish this, following 6-week Ni exposure, we isolated single cells and expanded the populations to obtain homogenous clones of Ni-washed-out cells (Ni-C) (see Methods for details) (Supplementary Figure S2). Gene expression analysis of six homogenous clones (Ni-C1 to Ni-C6) was performed using RNA-Seq. Principal component analysis (PCA) showed clustering of all the homogenous clones (Ni-C1 to Ni-C6), as well as heterogeneous population of Ni-washed-out cells (Figure 1D), signifying transcriptional similarity. This suggests that the effect of Ni is similar in all the exposed cells. Moreover, similar to Ni-washed-out cells (Figure 1B), all the six Ni-C cells showed EMT as one of the top enriched pathways (Figure 1E). These results suggest induction of EMT in all Ni-exposed BEAS-2B cells.

3.2 Nickel-exposed cells undergo persistent epithelial-mesenchymal transition (EMT)

To confirm acquisition of mesenchymal phenotype in Ni-exposed cells, we selected two Ni-clones (Ni-C1 and Ni-C2) for a comprehensive examination. We first evaluated the expression levels of epithelial markers, E-cadherin (CDH1) and claudin 1 (CLDN1), and mesenchymal marker, fibronectin 1 (FN1). Our results show downregulation of CDH1 and CLDN1 and upregulation of FN1 in Ni-C cells, compared to untreated control cells in both mRNA (Figure 2A) and protein levels (Figure 2B), suggesting EMT in Ni-exposed cells. Increased invasiveness is a hallmark of EMT. In addition, enhanced migratory abilities have also been observed in cells that have acquired mesenchymal phenotype. Therefore, we next compared the invasive and migratory abilities of Ni-C cells with that of the untreated control

cells. Transwell invasion assays revealed robust increase in the rate of invasion in Ni-C cells (Figure 2C, D). Furthermore, wound-healing assays showed increased migration in Ni-C cells compared to control cells (Figure 2E, F). Taken together, these results suggest that Ni exposure induced EMT and that the mesenchymal phenotype persisted even after the cells have proliferated for a number of generations, post Ni exposure (Ni-C cells). Next, we asked if persistent EMT induction happens in cells exposed to lower doses of Ni. To answer this, we exposed BEAS-2B cells to 10 μ M and 50 μ M NiCl₂ for 6 weeks. Following exposure, the cells were washed and cultured in NiCl₂-free medium for 2 weeks. As shown in Figure 2G, we found decreased CDH1 levels in the cells exposed to lower Ni concentrations, suggesting that Ni exposure could induce persistent EMT at lower doses.

3.3 Chronic Ni exposure is required for persistent EMT

Our results suggest that chronic Ni exposure could induce persistent EMT (Figure 2). We next asked whether short-term, high-dose Ni exposure could also induce EMT. To answer this question, we exposed BEAS-2B cells to 500 μ M NiCl₂ for 72 h (acute Ni-exposed). Following exposure, the cells were washed and cultured for two weeks without NiCl₂ (acute Ni-washed-out). RNA-Seq analysis showed that a smaller fraction of genes was persistently differentially expressed following acute Ni exposure (Supplementary Figure S3A), compared to chronic Ni exposure (Figure 1A). Furthermore, the gene expression profiles of acute Ni-exposed and acute Ni-washed-out cells did not cluster, suggesting transcriptional dissimilarity (Supplementary Figure S3A). This suggested that upon termination of acute Ni exposure, most of the differentially expressed genes reverted to the expression levels in untreated cells. Interestingly, pathway analysis of the transiently differentially expressed genes revealed EMT to be one of the top enriched pathways (Supplementary Figure S3B). However, the persistently differentially expressed genes did not show any association with EMT (Supplementary Figure S3C). These results suggest that although acute exposure to high doses of Ni could potentially initiate EMT, it may not persist after cessation of exposure.

3.4 Ni exposure induces invasiveness in non-invasive human cancer cells

Our results show that Ni exposure could induce persistent EMT in the non-invasive, non-malignant BEAS-2B cells and convert them to an invasive phenotype. We next asked if Ni could induce EMT in non-invasive cancer cells. To examine this, we exposed the non-invasive, RT4 human cancer cell line to 100 μ M NiCl₂ for 6 weeks. Following exposure, the cells were washed and cultured for 2 weeks in NiCl₂-free medium (Ni-washed-out). Similar to BEAS-2B cells, the Ni-exposed RT4 cells showed decreased CDH1 and CLDN1 levels and increased FN1 levels, which persisted after the cessation of exposure (Supplementary Figure S4A). In addition, loss of colonial morphology, formation of lamellipodia-like structures and increased cell spreading was seen in Ni-exposed cells (Supplementary Figure S4B). Furthermore, the Ni-exposed cells displayed increased invasive abilities (Supplementary Figure S4C, S4D). These results suggest that Ni exposure could induce EMT in RT4 cells. Therefore, induction of persistent EMT following Ni exposure is likely a trait shared by several cell-types.

3.5 ZEB1, a master regulator of EMT, is highly upregulated upon Ni exposure

To obtain mechanistic insights into the acquisition of persistent mesenchymal phenotype by Ni exposure, we examined the expression levels of genes within the EMT signaling pathway (Figure 1E). As shown in Figure 3A, we found *Zinc Finger E-Box Binding Homeobox 1* (*ZEB1*) to be one of the most highly upregulated genes in all the exposed cells (Ni-exposed, Ni-washed-out and Ni-washed-out homogenous clones, Ni-C1 to Ni-C6), compared to the levels in untreated cells. Evaluation of *ZEB1* mRNA (Figure 3B) and protein levels (Figure 3C) in Ni-C1 and Ni-C2 cells confirmed persistent upregulation of ZEB1 by Ni exposure.

ZEB1 is a negative regulator of CDH1 [41,42]. Several earlier studies have implicated ZEB1-mediated downregulation of CDH1 in the establishment of EMT [41,42]. Our identification of ZEB1 as the most upregulated gene in Ni-exposed as well as Ni-washed-out cells (Figure 3A) suggested that persistent upregulation of ZEB1 could play an important role in the acquisition of EMT in Ni-exposed cells.

3.6 ZEB1 depletion reverts Ni-induced EMT

To obtain insights into the role of ZEB1 in the establishment of Ni-induced EMT, we stably knocked-down ZEB1 in Ni-C1 and Ni-C2 cells using shRNAs (shZEB1). As shown in Figure 3D, ZEB1 depletion resulted in the recovery of CDH1 protein, reminiscent of mesenchymal-epithelial transition (MET). We next examined the invasive and migratory abilities of shZEB1 cells. Transwell invasion and wound healing assays showed that ZEB1 depletion caused decreased invasion (Figure 3E, F) and decreased migration (Figure 3G, H) in Ni-C1 and Ni-C2 cells. These results suggest Ni-induced EMT to be ZEB1-dependent. Interestingly, in addition to *ZEB1*, Ni exposure caused persistent upregulation of other EMT master regulators, *SNAIL* and *TWIST1*, although the upregulation was not as dramatic as *ZEB1* (Figure 3A). As shown in Figure 3D, ZEB1 depletion in Ni-C cells did not have any significant effect on the levels of SNAIL and TWIST1. Therefore, reversal of EMT in ZEB1-depleted cells, in spite of high levels of SNAIL and TWIST1 further substantiates ZEB1 as the major mediator of Ni-induced EMT.

In light of the significant role of ZEB1 in Ni exposure-induced EMT, we hypothesized that Ni exposure may not induce EMT in cells lacking ZEB1. To test this, we stably knocked down ZEB1 in untreated BEAS-2B cells using shRNAs (Figure 3I). The ZEB1-depleted cells (shZEB1) and the corresponding control cells (shControl) were then exposed to 100 μ M NiCl₂ for 6 weeks. Ni exposure did not cause CDH1 downregulation in shZEB1 cells (Figure 3I), although the shControl cells showed significant loss of CDH1 (Figure 3I), as expected. These results suggest that ZEB1 upregulation is required for EMT induction upon Ni exposure. Interestingly, Ni exposure increased the levels of SNAIL and TWIST1 in both shControl as well as shZEB1 cells (Figure 3I), indicating that ZEB1 knockdown did not affect Ni-induced upregulation of SNAIL and TWIST1 (Figure 3I). This further suggests that upregulation of ZEB1 is essential for EMT induction in BEAS-2B cells and that the increased expression of the other EMT master regulators could not compensate for the loss of ZEB1.

3.7 Hypoxia does not upregulate ZEB1 persistently

Since our results suggest a central role for persistent ZEB1 upregulation in Ni-induced EMT, we next wanted to gain insights into the molecular mechanisms associated with ZEB1 upregulation. *ZEB1* gene possesses four hypoxia response elements (HREs) at its proximal promoter [43]. HIF1 α has been shown to directly bind *ZEB1* HRE-3 and increase its expression [43]. Ni compounds are hypoxia mimetics, which could cause HIF1 α stabilization even under normoxia [44]. Therefore, we wanted to examine if the increase in HIF1 α levels, due to the hypoxia-like effect of Ni exposure could potentially play a role in ZEB1 upregulation and subsequent EMT. To accomplish this, we cultured untreated BEAS-2B cells in hypoxia (3% O₂) for 2 weeks. As expected, we found increase in the levels of HIF1 α protein in hypoxic cells (Figure 4A). We also found upregulation of ZEB1, suggesting that hypoxia can activate ZEB1 (Figure 4A). We next asked whether hypoxia-induced ZEB1 upregulation would continue even when the cells in hypoxia are subsequently reverted to normoxia (21% O₂). To answer this question, after two weeks in hypoxia, the cells were cultured in normoxia for two more weeks and ZEB1 levels were examined. Interestingly, in the cells that were reverted to normoxia, ZEB1 levels decreased to levels in the cells that were never exposed to hypoxia (Figure 4A). In contrast, cells exposed to 100 μ M NiCl₂ for the same period (2 weeks) showed persistent increase in ZEB1 levels even after being cultured without Ni for two more weeks (Ni-washed-out) (Figure 4B). This shows that that unlike Ni exposure, hypoxia could only transiently upregulate ZEB1. Together, these results suggest that the Ni-induced hypoxia-like effect may not be responsible for persistent ZEB1 upregulation.

3.8 ZEB1 is persistently activated by Ni-induced epigenetic alterations

Our earlier studies showed extensive epigenetic changes in Ni-exposed cells [38]. Therefore, we next wanted to test if there is an epigenetic basis for Ni-induced persistent upregulation of ZEB1. To accomplish this, we examined the chromatin environment of *ZEB1* gene promoter by assessing the levels of histone modifications associated with gene activation (H3K4me3) and repression (H3K27me3) using chromatin immunoprecipitation (ChIP) assays. Our results show enrichment of H3K4me3 (Figure 5A), as well as H3K27me3 (Figure 5B) at *ZEB1* promoter in untreated BEAS-2B cells, suggesting poised bivalent chromatin configuration. Interestingly, in Ni-C cells, although H3K4me3 levels remained the same as that of untreated cells (Figure 5A), H3K27me3 levels decreased significantly (Figure 5B). This indicates resolution of epigenetic bivalency at *ZEB1* promoter to monovalency by Ni exposure. These results suggest that in untreated BEAS-2B cells, the bivalent chromatin at *ZEB1* promoter suppresses ZEB1 expression, thus maintaining the cells in an epithelial state. Conversion of repressive bivalent chromatin to active chromatin configuration by Ni exposure, via loss of H3K27me3 increases ZEB1 expression, resulting in the cells switching to mesenchymal state. Interestingly, epigenetic monovalency persisted even after the cessation of exposure (Figure 5A, B).

Members of microRNA (miR), *miR-200* family genes and *miR-205* are *ZEB1*, repressors [45]. On the other hand, ZEB1 can downregulate *miR-200/205* expression by binding to their promoters and recruiting transcriptional repressors [46]. Therefore, we next asked whether miRNAs could potentially be associated with Ni-induced persistent

ZEB1 upregulation. As shown in Figure 5C, we found persistent downregulation of *miR-200a*, *miR-200b*, *miR-200c* and *miR-205* in Ni-C cells. Interestingly, upon ZEB1 depletion, the expression of miRNAs was restored to that of control levels (Figure 5C). These results suggest that ZEB1, upregulated by Ni exposure, could suppress the expression of its repressors *miR-200/205*, which likely resulted in increased and sustained upregulation of ZEB1, causing persistent EMT. Taken together, our results suggest that epigenetic activation of ZEB1 plays a key role in the Ni-induced acquisition of persistent mesenchymal state.

4 DISCUSSION

In this study, we examined the persistent effects of exposure to Ni, an environmentally prevalent, low-mutagenic carcinogen. We report that chronic low-dose Ni exposure induces transcriptional changes that persist even after the termination of exposure, in a surprisingly high number of genes. Through comprehensive examination of the irreversible effects of Ni exposure, we show that the Ni-exposed cells undergo epithelial-mesenchymal transition (EMT) and that the mesenchymal state continues even after the cessation of exposure.

During EMT, cells lose their junctions and polarity, and become migratory and invasive [29]. EMT is an essential cellular process involved in developmental events such as heart and neural crest development, and wound healing [47,48]. However, EMT is also a major pathogenic process associated with airway remodeling in asthma, fibrosis [29], development of premalignancy [49], cancer progression and metastasis [47,48]. This suggests that the persistent transcriptional alterations and EMT induction that we have identified in Ni-exposed cells could be important in the development of Ni exposure-associated diseases.

Our results show that Ni exposure causes persistent decrease in the levels of E-cadherin (CDH1), a cell-cell adhesion epithelial protein involved in the maintenance of tissue architecture [50]. Decreased CDH1 levels is associated with cellular dedifferentiation, decreased cell-cell contacts, and increased migratory and invasive capabilities [51], characteristic traits of mesenchymal cells. Therefore, functional loss of CDH1 is considered a hallmark of EMT [51]. Ni-induced CDH1 downregulation has been observed in earlier studies [52], suggesting CDH1 as a Ni target, although persistence of CDH1 downregulation and EMT has not been investigated thus far. A number of repressors are known to mediate CDH1 downregulation. Key among the CDH1 repressors is a group of proteins identified as EMT master regulators, which include ZEB, SNAIL and TWIST families [41,53].

Among the EMT associated genes, we found *ZEB1* to be the top upregulated, both during Ni exposure and in all the Ni-clones (Ni-C1 to Ni-C6) (Figure 3A). *ZEB1* is activated early in EMT and plays a central role in fibrosis and cancer development [43,47,48]. *ZEB1* directly binds E-box sequences of *CDH1* gene promoter and represses it by altering its chromatin environment through recruitment of DNMT1, CtBP and BRG1 [42,54,55]. In addition, *ZEB1* downregulates epithelial polarity genes and upregulates invasion and metastasis associated genes [43,48]. Therefore, increased expression of *ZEB1* is implicated in EMT induction [43,53]. Consequently, *ZEB1* expression has been associated with aggressive epithelial tumor types and poor prognosis [56,57]. Interestingly, *ZEB1* depletion in

mesenchymal cells has been shown to be sufficient to restore CDH1 levels and re-establish epithelial phenotype [58,59]. Consistent with this, our results show that ZEB1 depletion in Ni-clones could restore CDH1 levels and revert the cells back to epithelial phenotype (Figure 3D–H), suggestive of mesenchymal-epithelial transition (MET). Furthermore, Ni exposure did not induce EMT in ZEB1 depleted cells (Figure 3I). Collectively, these results suggest a major role for ZEB1 upregulation in Ni-induced EMT.

In addition to *ZEB1*, we observed persistent upregulation of EMT master regulators *SNAIL* and *TWIST1* in Ni-exposed cells, although the increased expression was not as robust as that of *ZEB1* (Figure 3A). This suggests the possibility of these EMT master regulators being involved in Ni-induced EMT. However, the ZEB1-depleted cells, which have undergone reversion of mesenchymal phenotype and display epithelial characteristics, still retained high levels of SNAIL and TWIST1 (Figure 3D). Moreover, exposure of ZEB1-depleted cells to Ni did not downregulate CDH1, in spite of significant upregulation of SNAIL and TWIST1 (Figure 3I). Taken together, these results suggest that upregulation of ZEB1, and not the other EMT master regulators, is key for Ni-induced persistent EMT. These results are particularly interesting since SNAIL, is a stronger repressor of CDH1, compared to ZEB1 [60]. Nevertheless, it has to be remembered that SNAIL and TWIST are capable of activating ZEB1 [61]. Therefore, we cannot rule out the possibility of SNAIL and TWIST acting upstream of ZEB1 during EMT induction.

Examination of the chromatin environment at *ZEB1* promoter in untreated BEAS-2B cells revealed enrichment of both H3K4me3 and H3K27me3 (Figure 5A, B), histone modifications associated with gene activation and repression, respectively. This suggests bivalent chromatin configuration at *ZEB1* promoter. A recent study has also suggested bivalency at *ZEB1* promoter in human breast cancer cells [62]. Bivalent chromatin is known to maintain genes in a repressed state, while poising them for rapid activation upon receiving appropriate stimuli [63]. We found that Ni exposure resolved the repressive bivalent chromatin at *ZEB1* promoter to active monovalent chromatin through the loss of H3K27me3. Interestingly, the monovalent chromatin persisted even after the termination of Ni exposure, resulting in persistent ZEB1 upregulation. Moreover, ZEB1 is capable of self-activation [64], which likely contributes to its own upregulation. Although how Ni exposure causes decrease in H3K27me3 levels at *ZEB1* promoter remains unknown, our results suggest resolution of bivalent chromatin at *ZEB1* promoter as an important event that leads to persistent EMT upon Ni exposure (Figure 6).

The *miR-200* family genes, *miR-200a*, *miR-200b* and *miR-200c*, as well as *miR-205* directly target *ZEB1* mRNA and therefore are important repressors of *ZEB1* [45]. Conversely, ZEB1 protein can target *miR-200* family and *miR205* promoters causing their downregulation [46]. Thus, *miR-200/205* and ZEB1 regulate each other's expression through a double negative feedback loop [46,65]. While ZEB1 downregulation by miRNAs is associated with epithelial phenotype, its upregulation caused *miR-200/miR205* repression and EMT induction [45,46,65]. Thus, it is clear that the alterations in the levels of ZEB1 and *miR-200* family/*miR205* could switch cells between epithelial and mesenchymal phenotypes [46,65]. Our results show that Ni-induced upregulation of ZEB1 caused persistent downregulation of its repressors, *miR-200a*, *miR-200b*, *miR-200c* and *miR-205* (Figure 5C).

This suggests that ZEB1 upregulation caused by Ni exposure could disrupt the ZEB1-miRNA double-negative feedback loop, resulting in sustained ZEB1 upregulation, which contributes to the acquisition of persistent mesenchymal phenotype (Figure 6).

Cancer development is a multistep process. During carcinogenesis, the cells evolve from normal into invasive cancers through a number of pre-malignant states [66]. EMT, a key feature of metastatic cells has long been thought of as the last step in cancer progression. However, through recent studies, the role of EMT induction in cancer initiation is beginning to be understood [49,67]. Moreover, growing evidence on cell dissemination from premalignant lesions suggests that metastasis is not always an end point of cancer development, but can be an early event in disease initiation [49,68,69]. Besides cancer, EMT is also implicated in other chronic lung diseases associated with Ni exposure such as airway remodeling in asthma [70], and pulmonary fibrosis [71]. Therefore, our finding of persistent EMT induction caused by Ni exposure through irreversible epigenetic activation of ZEB1 could be important in the understanding of Ni-induced diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ATCC	American type culture collection
CDH1	E-cadherin
ChIP	chromatin immunoprecipitation
CLDN1	claudin 1
DMEM	Dulbecco's modified Eagle's medium
EMT	epithelial-mesenchymal transition
FBS	fetal bovine serum
FN1	fibronectin 1
GAPDH	glyceraldehyde phosphate dehydrogenase
GEO	gene expression omnibus
HREs	hypoxia response elements
IARC	International agency for research on cancer

MET	mesenchymal-epithelial transition
miR	microRNA
Ni-C	homogenous clones of Ni-washed-out cells
PCA	principal component analysis
qPCR	quantitative PCR
RPKM	reads per kilobase of exon per million fragments mapped
ZEB1	zinc finger E-box binding homeobox 1

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FIGURE 1. Nickel exposure causes persistent gene expression changes

BEAS-2B cells were treated with 100 μ M NiCl₂ for 6 weeks (Ni-exposed). After exposure, the cells were grown in Ni-free medium for 2 weeks (Ni-washed-out). Gene expression was examined using RNA-Seq. (A) RNA-Seq results are displayed as heatmap showing the persistently and transiently differentially expressed genes (2-fold up- or down- regulation). Log2 transformed RPKM values were used to generate the heatmap. (B, C) Functional enrichment analysis showing the major pathways associated with the persistently (B), and transiently (C) differentially expressed genes. (D) Principal Component Analysis (PCA) shows that the gene expression profiles of all the Ni-washed-out homogenous clones (Ni-C1 to Ni-C6) are similar. Ni washed-out heterogeneous cells (Ni-W) also cluster with Ni-C cells suggesting transcriptional similarity. Control: untreated cells; Ni-E: Ni-exposed cells; Ni-W: Ni-washed-out heterogeneous cells; Ni-C1 to Ni-C6: Ni-washed-out homogenous clones. (E) Functional enrichment analysis (IPA) of the persistently differentially expressed genes in all the six homogenous clones (Ni-C1 to Ni-C6) shown as heatmap.

FIGURE 2. Chronic Ni exposure induces epithelial-mesenchymal transition (EMT)

(A, B) qPCR analysis (A), and western blotting analysis (B) showing downregulation of epithelial markers CDH1 and CLDN1, and upregulation of mesenchymal marker FN1 in Ni-C1 and Ni-C2 cells. For qPCR analysis, *GAPDH* was used as internal control. For western blotting analysis, β actin was used as loading control. (C, D) Invasion assay showing increased invasive ability of Ni-C cells compared to untreated control cells. Representative images (10x) (C), and quantification of cell invasion performed in duplicates by counting invaded cells from four fields in each insert (D). (E, F) Wound healing assay showing increased rate of migration in Ni-C cells compared to untreated control cells. Representative images (10x) (E), and quantification of wound healing shown as percentage of wound closure 24 h after scratch (F). (G) Western blotting analysis showing downregulation of CDH1 in BEAS-2B cells exposed to various doses of NiCl₂. All error bars represent standard deviations from at least two biological replicates. Statistical significance was evaluated using t-test ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)).

FIGURE 3. EMT master regulator *ZEB1* plays a central role in Ni exposure-induced persistent EMT

(A) Heat map showing the expression levels of genes within the "Regulation of EMT pathway" (IPA) in Ni-washed-out homogenous clones (Ni-C1 to Ni-C6). *ZEB1* is the top upregulated gene in all the Ni-C cells. (B) qPCR analysis showing mRNA levels of *ZEB1* in untreated control and Ni-C cells. *GAPDH* was used as internal control (C) Western blotting analysis showing *ZEB1* protein levels in untreated control and Ni-C cells. β actin was used as loading control. (D) Stable knockdown of *ZEB1* restores *CDH1* levels in Ni-C cells. *SNAIL* and *TWIST1* levels were not significantly altered by *ZEB1* knockdown. β actin was used as loading control. (E, F) Invasion assay showing decreased invasive ability of Ni-C

cells upon ZEB1 knockdown. Representative images (10x) (E), and quantification of cell invasion performed in duplicates by counting invaded cells from four fields in each insert (F). (G, H) Wound healing assay showing decreased rate of migration in Ni-C cells upon ZEB1 knockdown. Representative images (10x) (G), and quantification of wound healing shown as percentage of wound closure 24 h after scratch (H). (I) CDH1 is downregulated in BEAS-2B cells exposed to Ni (shControl). However, in cells with stable ZEB1 knockdown (shZEB1), Ni exposure did not downregulate CDH1. SNAIL and TWIST1 are upregulated in both shControl and shZEB1 cells upon Ni exposure. β actin: loading control. All error bars represent standard deviations from at least two biological replicates. Statistical significance was evaluated using t-test ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)).

FIGURE 4. Hypoxia does not cause persistent ZEB1 upregulation

(A) Western blotting analysis showing HIF1α and ZEB1 protein levels in BEAS-2B cells. Control (normoxia): cells cultured at 21% O₂ (atmospheric O₂ levels) for 2 weeks; Hypoxia: cells cultured at 3% O₂ for 2 weeks; Normoxia Revert: cells cultured at 3% O₂ for 2 weeks, followed by culturing at 21% O₂ for 2 weeks. While significant increase in HIF1α and ZEB1 protein levels occurred under hypoxia, subsequent reverting the cells to normoxia reduced their levels to that of control cells that were never exposed to hypoxia. (B) Western blotting analysis showing HIF1α and ZEB1 protein levels in BEAS-2B cells. All the cells were cultured at 21% O₂ (atmospheric O₂ levels). Untreated: untreated cells; Ni-exposed: cells exposed to Ni for 2 weeks; Ni-washed-out: cells exposed to Ni for 2 weeks and subsequently cultured in Ni-free medium for 2 more weeks. Both HIF1α and ZEB1 protein levels increased in Ni-exposed cells. While HIF1α levels decreased upon Ni-wash-out, ZEB1 levels remained persistently high.

FIGURE 5. ZEB1 is epigenetically activated by Ni exposure

(A) ChIP-qPCR analysis showing enrichment of H3K4me3 at *ZEB1* promoter in BEAS-2B cells. High levels of H3K4me3 is seen in untreated cells and the levels are unaltered in Ni-C cells. ChIP-qPCR results shown as fold enrichments of H3K4me3 over IgG. Error bars correspond to standard deviations from at least two biological replicates. (B) ChIP-qPCR analysis showing enrichment of H3K27me3 at *ZEB1* promoter in BEAS-2B cells. High levels of H3K27me3 are seen in untreated cells and significant reduction in H3K27me3 levels occurred in Ni-C cells. ChIP-qPCR results shown as fold enrichments of H3K27me3 over IgG. Error bars correspond to standard deviations from at least two biological replicates. (C) Analysis of miRNA expression. qPCR analysis showing downregulation of miRNAs associated with EMT in Ni-C1 and Ni-C2 cells, compared to their levels in untreated cells. In Ni-C cells, stable ZEB1 knockdown (Ni-C1-shZEB1 and Ni-C2-shZEB1) resulted in restoration of the miRNA expression levels to that of the control cells. *SNORD44* was used as internal control. The mRNA levels of untreated control cells, Ni-C1-shControl and Ni-C2-shControl cells were normalized to 1. All error bars correspond to standard deviations from at least two biological replicates. Statistical significance was evaluated using t-test ($p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***)). NS: Not significant.

FIGURE 6. Proposed model for Ni-induced persistent EMT

In untreated BEAS-2B cells, strong enrichment of both H3K4me3 and H3K27me3 at *ZEB1* promoter suggests repressive bivalent chromatin configuration, which potentially maintains its expression at low levels. *miR-200* family genes and *miR-205*, repressors of *ZEB1*, likely contribute to the low levels of *ZEB1* expression. This results in high E-cadherin (*CDH1*) expression and maintenance of the cells in an epithelial state. Ni exposure results in the reduction of H3K27me3 levels at *ZEB1* promoter, while retaining high H3K4me3 levels, thereby resolving the repressive bivalent chromatin to monovalent active configuration. Levels of H3K27me3 remain low even after the exposure has ceased, thus activating *ZEB1* transcription in a persistent manner. Moreover, the ability of *ZEB1* to activate itself could further enhance its expression levels. In addition, *ZEB1* protein targets its negative regulators, *miR-200* family genes and *miR-205*, causing their downregulation, thus disrupting the *ZEB1*-miRNA double-negative feedback loop. Therefore, (i) resolution of bivalent chromatin configuration at *ZEB1* promoter, (ii) self-activation of *ZEB1*, and (iii) downregulation of negative regulators of *ZEB1*, could collectively result in *ZEB1* expression becoming self-sustaining and persistent even after the cessation of Ni exposure. The persistently increased *ZEB1* protein causes *CDH1* repression. Consequently, the cells undergo EMT and acquire mesenchymal traits including increased invasion and migration. Orange ovals denote H3K27me3 and yellow ovals denote H3K4me3. Silent or active status of *ZEB1* gene is represented by broad red and green arrows, respectively. Thick solid red lines denote strong repression and thin solid red lines denote weak repression. Blue, up and down arrows denote higher and lower expression, respectively.