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Cadmium exposure upregulates SNAIL through miR-30 repression in human lung epithelial cells

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Abstract

Cadmium (Cd) is a known human lung carcinogen. In addition, Cd exposure is associated with several lung diseases including emphysema, chronic obstructive pulmonary disease (COPD), asthma and fibrosis. Although earlier studies have identified several processes dysregulated by Cd exposure, the underlying mechanisms remain unclear. Here, we examined the transcriptome of lung epithelial cells exposed to Cd to understand the molecular basis of Cd-induced diseases. Computational analysis of the transcriptome predicted a significant number of Cd-upregulated genes to be targets of miR-30 family miRNAs. Experimental validation showed downregulation of all the miR-30 family members in Cd exposed cells. We found SNAIL, an EMT master regulator, to be the most upregulated among the miR-30 targets. Furthermore, we found decrease in the levels of epithelial marker E-cadherin (CDH1) and increase in the levels of mesenchymal markers, ZEB1 and vimentin. This suggested induction of EMT in Cd exposed cells. Luciferase reporter assays showed that miR-30 repressed SNAIL by directly targeting its 3' UTR. Over expression of miR-30e and miR-30 mimics reduced Cd-induced SNAIL upregulation. Our results suggest that miR-30 negatively regulates SNAIL in lung epithelial cells and that Cd-induced downregulation of miR-30 relieves this repression, resulting in SNAIL upregulation and EMT induction. EMT plays a major role in many diseases associated with Cd exposure including fibrosis, COPD and cancer and metastasis. Therefore, our identification of miR-30 downregulation in Cd exposed cells and the consequent activation of SNAIL provides important mechanistic insights into lung diseases associated with Cd exposure.

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Conflicts of Interest

The authors declare no competing financial interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords

Cadmium; SNAIL; epigenetics; miR-30; EMT; lung epithelial cells; miRNAs

Introduction

Cadmium is a toxic metal found naturally in the environment. Apart from natural processes like forest fires and volcanic activity, anthropogenic sources like fuel burning, welding, smelting, manufacturing of pigments and Ni-Cd batteries increase this pollutant in the environment (Hutton, 1983; Pinot et al., 2000; Bernhoft, 2013). Humans are exposed to Cd through contaminated air, water, food and cigarette smoke (Pinot et al., 2000; IARC., 2012; Bernhoft, 2013). Occupational exposure is another major source of Cd exposure, especially in workers involved in pigment industries and Ni-Cd battery manufacture (IARC., 2012). Upon absorption, Cd is taken up by metal transporters on the plasma membrane and sequestered by cytosolic metallothionein proteins and finally accumulate in organs such as liver, kidney, prostate and lung due to its long half-life and poor clearance mechanism (Klimisch, 1993; Klaassen and Liu, 1998; Sabolic et al., 2010; IARC., 2012).

Cd is a carcinogen, associated with cancers of the lung, kidney, pancreas, urinary and breast in humans (Huff et al., 2007; Wang et al., 2012; Nawrot et al., 2015). Inhalation is a major route of Cd exposure in humans and consequently lung is an important target organ. Lung cancer is by far the leading cause of cancer deaths among both men and women, with cigarette smoking being a major risk factor (Bray et al., 2018). Cd is one of the most potent carcinogens present in tobacco smoke. Furthermore, inhalation exposure to Cd causes increased oxidative stress, lung inflammation, emphysema, reduced lung function chronic obstructive pulmonary disease (COPD), asthma and lung fibrosis (Nemery, 1990; Leduc et al., 1993; Howard and Billings, 2000; Rennolds et al., 2010; Blum et al., 2014; Richter et al., 2017; Ganguly et al., 2018). Therefore, Cd exposure is a major human health hazard.

Earlier studies have identified several molecular processes that are dysregulated by Cd exposure. These include induction of oxidative stress, inhibition of apoptosis and DNA repair process (Sherrer et al., 2018). In addition, dysregulation of epigenetic processes including DNA methylation and histone acetylation causing aberrant gene transcription has been associated with Cd exposure (Park et al., 2017; Peng et al., 2019; Wang and Yang, 2019). Various molecular signaling cascades induced by Cd exposure are known. For example, Cd exposure activates KRAS dependent RAS/ERK signaling, leading to transformation of prostate epithelial cells (Ngalame et al., 2016). Furthermore, chronic exposure to Cd also resulted in cancer stem cell like property and epithelial-mesenchymal transition (EMT) phenotype leading to transformation in immortalized human pancreatic ductal epithelial cells (Qu et al., 2012). However, the molecular mechanisms underlying Cd-induced alterations to biological processes are still not well understood.

Recent investigations have focused on studying global transcriptomic changes to understand the molecular basis of environmental diseases (Luparello et al., 2011; Jose et al., 2018). Leveraging omics data such as transcriptomics and epigenomics allows development of an integrated perspective of genetic, epigenetic and signaling network cross talk on cellular

response or disease progression due to environmental exposures. Compilation of large curated mechanistic databases and computational prediction methods have made it possible to understand the sequence of events using genome-wide data sets and generate hypotheses that can be validated using experimental approaches (Kramer et al., 2014).

Here, we analyzed the transcriptome of Cd exposed lung epithelial cells. Using computational prediction followed by experimental validation, we show that Cd exposure downregulates miR-30 family of miRNAs. miR-30 downregulation by Cd exposure caused induction of the transcription factor, SNAIL. SNAIL is a master regulator of epithelial-mesenchymal transition (EMT), a process in which polarized epithelial cells lose cell-cell adhesion and acquire invasive and migratory mesenchymal properties (Cano et al., 2000). Increased SNAIL expression is associated with cancer, metastasis, cancer sternness, and increases tumor recurrence (Cano et al., 2000; Deep et al., 2014). In addition, SNAIL expression and EMT is implicated in several Cd-induced lung diseases including pulmonary fibrosis and COPD (Milara et al., 2013; Lamouille et al., 2014). Therefore, our studies showing miR-30 downregulation by Cd provides major insights into pathogenesis associated with Cd exposure.

Materials and methods

Cell culture and treatments

Human lung epithelial BEAS-2B and BEP2D cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro, Mediatech, Corning, Manassas, VA) supplemented with 1% Penicillin-Streptomycin and 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Flowery Branch, GA) at 37°C and 5% CO₂. For Cd exposure, the cells were treated with 0, 2.5, 5 and 10 µM CdCl₂ (AC315271000, Fisher Scientific) for 72 h. To investigate the time-dependent effects of Cd exposure (Fig. 3 and 5), BEAS-2B cells were treated with 10 µM CdCl₂ for the specified periods (0, 1, 3, 6, 12 and 24 h).

Cytotoxicity assay

Cytotoxicity of Cd in BEAS-2B cells was examined using Promega CellTiter 96 non-radioactive cell proliferation assay kit (G4001), according to manufacturer's instructions. Five thousand cells were seeded in triplicates in 96-well plates and incubated at 37°C for 24 h. The cells were then treated with 0–10 µM CdCl₂ for 72 h. Cytotoxicity was measured at 24, 48 and 72 h time-points.

RNA isolation and qPCR

Total RNAs were isolated using RNeasy kit (Qiagen). miRNAs were isolated using miRNA isolation kit (Qiagen). cDNA synthesis for protein coding genes and miRNA genes were 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 FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Indianapolis, IN) on a 7900HT Fast Real-Time PCR system (Applied Biosystems). *GAPDH* and *SNORD44* were used as internal controls for protein coding and

miRNA genes, respectively. Experiments were performed in duplicate. Statistical significance of qPCR results was evaluated using *t*-test.

RNA-Seq and data analysis

RNA samples from two biological replicates were used for RNA-Seq analysis. 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, as explained earlier (Kartashov and Barski, 2015; Jose et al., 2018). 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 and genes that show fold change ≥ 1.5 and $\text{padj} < 0.05$ were considered as differentially expressed. Volcano plot was generated using R (<http://www.R-project.org/>). RNA-Seq data were submitted to Gene Expression Omnibus (GEO) under the accession number GSE128263.

Gene ontology (GO), upstream regulator and miRNA enrichment analysis

Gene enrichment analysis was carried out using functional enrichment analysis tool, FunRich 3.1.3 (Pathan et al., 2015; Pathan et al., 2017). Differentially expressed genes (fold change ≥ 1.5 ; $\text{padj} < 0.05$) were used to identify the enriched GO biological processes. The statistical significance of enriched terms was evaluated by hypergeometric distribution test corrected using FDR method. Upstream Regulator Analysis (URA) was used to identify the upstream regulators associated with the differentially expressed genes. The enrichment scores (Fisher's exact test *p*-values) along with the *z*-scores were used for ranking the top upstream regulators (Kramer et al., 2014). Enrichment of miRNA targets in the differentially expressed gene list was analyzed using ToppGene suite (Chen et al., 2009). To predict miRNA binding at the 3' UTR of differentially expressed genes, ToppGene uses multiple algorithms and databases including microRNA.org, PITA, TargetScan and miRTarbase. *q*-value FDR $B \& Y < 0.05$ was used for determining statistical significance (Benjamini, 2001; Lewis et al., 2005; Chen et al., 2009).

Western blotting

The total proteins were isolated using Laemmli buffer (Bio-Rad, Hercules, CA) or RIP A buffer. The proteins separated on SDS-PAGE gels were transferred to nitrocellulose membranes (Bio-Rad) and probed with antibodies against SNAIL (3879, Cell Signaling, Danvers, MA), ZEB1 (3396, Cell Signaling, Danvers, MA), CDH1 (NBP2–19051, Novus Biologicals, Littleton, CO), and Vimentin (5741P, Cell Signaling, Danvers, MA). Loading control: α -Tubulin (ab52866, Abcam, Cambridge, MA). The band intensities were quantified using ImageJ software.

Stable miR-30e overexpression

The miR-30e-5p overexpression plasmid (HmiR0007-MR04), and miRNA scrambled negative control plasmid (CmiR0001-MR04) were obtained from GeneCopoeia (Rockville, MD, USA). The plasmids were transfected using Lipofectamine 2000 reagent (Invitrogen)

following manufacturer's protocol. Briefly, 150,000 BEAS-2B cells were seeded in 6 well plates. After 24 h, 1 µg plasmids were transfected into BEAS-2B cells. After 48 h, 0.3 µg/ml puromycin was added for selection. The cells were selected for 3 weeks to obtain stable overexpression. miR30e-5p overexpression was confirmed using qPCR analysis.

Plasmid construction for luciferase assay

SNAIL 3' UTR reporter plasmids were constructed using pIS0 luciferase vector. pIS0 was a gift from David Bartel (Addgene plasmid #12178; <http://n2t.net/addgene:12178> ; RRID:Addgene_12178). *SNAIL* 3' UTR (820 bases) was amplified using the following oligonucleotides: Forward, 5' TATTGCTAGCTGACCCTCGAGGCTCCCT 3' and Reverse, 5' CCGCTGTCTAGACTGCTTTATTGAATATCA 3', and inserted into *NheI*/*XbaI* sites in the pIS0 vector (WT-SNAIL). Mutation at the miR-30e binding sequence in *SNAIL* 3' UTR was carried out using PCR mutagenesis using overlapping primers containing the desired mutation. The following oligonucleotides were used for PCR mutagenesis: 5' GGGCCTGGGAGGAAGACCCCGGGATTTTAAAGGTACAC 3' and 5' GTGTACCTTTAAAAATCCCGGGTCTTCCTCCCAGGCC 3' (mut-SNAIL). (Kumarswamy et al., 2012) The mutated bases are underlined.

For luciferase assay, BEAS-2B cells were seeded in a 24-well plate and transfected with WT-SNAIL or mut-SNAIL firefly luciferase constructs using lipofectamine 2000 (Invitrogen, CA, USA). A renilla luciferase plasmid (pIS2) was co-transfected for normalization. One day after transfection, the cells were either left untreated or treated with 10 µM Cd for 24 h. Firefly and renilla luciferase activities were assayed using the Dual Luciferase Reporter Assay System (Promega, WI, USA) and an LMax II 384 luminometer (Berthold Technologies, Bad Wildbad, Germany). Experiments were performed as two independent replicates.

For miR mimic experiments, BEAS-2B cells were seeded in a 24-well plate and transfected with the WT-SNAIL or mut-SNAIL firefly luciferase reporter plasmid together with miR hsa-miR-30e-5p mimic (miR mimic) or miRNA mimic Negative Control (Mimic-NC) (Dharmacon, IL, USA) at a final concentration of 40 pmol. A renilla luciferase plasmid (pIS2) was co-transfected for normalization. One day after transfection, the cells were treated with 10 µM Cd for 24 h. Firefly and renilla luciferase activities were assayed using the Dual Luciferase Reporter Assay System (Promega, WI, USA) as explained earlier.

Statistical analysis

Statistical significance of all q-PCR data was evaluated using *t*-test. Differential gene expression was calculated using DESeq2 and the genes that showed fold change ± 1.5 and *padj* <0.05 were considered as differentially expressed. GO biological process enrichment analysis of the differentially expressed genes was carried out using FunRich 3.1.3 tool and *p*-value <0.05 was considered significant. miRNA enrichment analysis was performed using Toppgene suite, and *q*-value false discovery rate (FDR) (Benjamini and Yekutieli [B&Y]) <0.05 (Benjamini, 2001) was considered significant.

Results

Exposure to cadmium affects cancer related biological processes

To investigate the consequences of cadmium exposure, human lung epithelial (BEAS-2B) cells were exposed to a noncytotoxic and environmentally relevant concentration of 5 μM CdCl_2 for 72 h (Supplementary Figure 1) (Mussalo-Rauhamaa et al., 1986; Jin et al., 2003). Global gene expression analysis of Cd-exposed cells was carried out using RNA-Seq. We found differential expression of 801 genes (fold change ≥ 1.5 ; $p_{\text{adj}} < 0.05$). As shown in Figure 1A, we found more genes to be upregulated in Cd exposed cells (613 upregulated and 188 downregulated). To gain insights into the functional consequences of Cd exposure, GO biological process enrichment analysis of the differentially expressed genes was carried out using FunRich 3.1.3 tool (Pathan et al., 2015; Pathan et al., 2017). Our analysis showed dysregulation of several processes, including regulation of cell proliferation, cytoskeletal reorganization, inflammatory response, and wound healing in the Cd exposed cells (Fig. 1B). This suggests that gene expression changes caused by Cd exposure might be related to cancer-associated processes. To investigate if Cd affected gene expression in a dose-dependent manner, we exposed BEAS-2B cells to 2.5, 5 and 10 μM CdCl_2 for 72 h. Examination of the mRNA levels of several candidate genes that were identified as differentially expressed by RNA-Seq analysis revealed a dose-dependent response to Cd exposure (Fig. 1C, D).

Cd exposure downregulates miR-30 family miRNAs

To obtain mechanistic insights into Cd-induced gene expression changes, the differentially expressed genes were subjected to ToppFun analysis of the ToppGene suite (Lewis et al., 2005; Chen et al., 2009). ToppFun performs functional enrichment analysis of a given gene list using several annotation categories including GO terms, pathways, protein-protein interactions, transcription factor-binding sites and microRNAs (miRNAs). Our analysis predicted a number of Cd- upregulated genes (307 out of 613 genes) to be targets of miRNAs ($q\text{-value FDR B\&Y} < 0.05$) (Fig. 2A). In contrast, we did not find any miRNA targets among the downregulated genes (Fig. 2A). Earlier studies have identified transcriptional alterations of miRNAs due to Cd exposure (Brooks et al., 2016; Brooks and Fry, 2017; Fay et al., 2018). However, systematic analyses of the differentially expressed miRNAs and their target genes have not been carried out thus far. Therefore, we next wanted to examine the effects of Cd-exposure on miRNAs and their functional consequences. Our analysis shows that the upregulated genes are targets of a number of miRNAs (Fig. 2B). Interestingly, 23.5% (72 genes) of the miRNA target genes in the upregulated gene set were targets of miR-30 family miRNAs (Fig. 2C). miRNAs bind the 3' UTR of their target genes and negatively regulate their expression by reducing mRNA levels or by decreasing translation efficiency. Downregulation of miRNAs causes upregulation of their targets. Therefore, our identification of a large number of miR-30 family targets among the Cd upregulated genes suggested that the downregulation of miR-30 family miRNAs could be an important pathogenic mechanism associated with Cd exposure. To test this, we examined the expression levels of miR-30 family miRNAs in BEAS-2B cells exposed to multiple doses of Cd. As expected, Cd exposure resulted in a dose-dependent downregulation of all the

miR-30 family genes, miR-30a, miR-30b, miR-30c-1, miR-30c2, miR-30d, and miR-30e (Fig. 2D).

Cd exposure induces SNAIL expression

miR-30 family miRNAs are tumor suppressors frequently found to be downregulated in multiple types of cancers including the cancers of lung (Kumarswamy et al., 2012; Yang et al., 2017; Mao et al., 2018). Our findings suggest that a large number of Cd-upregulated genes were targets of miR-30 family (Fig. 2C). We also found dose-dependent downregulation of all the miR-30 family members in Cd-exposed cells (Fig. 2D). These results indicate an important role for miR-30 family in Cd-induced pathogenesis. To understand the functional significance of miR-30 downregulation in Cd exposed cells, we examined the expression levels of the 72 genes predicted by our analysis to be the targets of miR-30 (Fig. 2C). Interestingly, our analysis showed *SNAIL*, the gene that encodes SNAIL protein, as the top upregulated among the miR-30 target genes (Fig. 3A). SNAIL is a zinc-finger transcription factor involved in cell differentiation and survival (Kaufhold and Bonavida, 2014). The most well characterized function of SNAIL is the induction of epithelial-mesenchymal transition (EMT) (Cano et al., 2000; Lamouille et al., 2014). Typically, SNAIL is expressed at low levels in the epithelial cells. Increased expression of SNAIL is associated with the epithelial cells acquiring mesenchymal characteristics including increased invasive and migratory potential. Therefore, SNAIL is considered a master regulator of EMT (Cano et al., 2000; Lamouille et al., 2014).

To confirm SNAIL upregulation in Cd exposed cells, we exposed BEAS-2B cells to multiple doses of Cd for 72 h. Examination of the mRNA levels showed dose-dependent increase in *SNAIL* expression (Fig. 3B). Validating this, western blotting analysis showed a corresponding dose-dependent increase in the SNAIL protein levels (Fig. 3C). In addition, we found a time-dependent increase in SNAIL levels in Cd exposed BEAS-2B cells. The levels of SNAIL quickly increased within 3 h of Cd exposure, reaching maximal protein levels at 12 h (Fig. 3D). These results validated our computational predictions and confirmed that SNAIL is upregulated by Cd exposure, in a dose-, and time-dependent manner.

In addition to increased expression of SNAIL, EMT induction is typically associated with repression of epithelial proteins, the most critical being E-cadherin (CDH1), a protein involved in cell-cell adhesion. Therefore, to further examine EMT induction in Cd exposed cells we assessed the levels of CDH1. Our results show decrease in CDH1 levels in the Cd exposed cells (Fig. 3E). In addition, we found upregulation of mesenchymal markers ZEB1 and vimentin (Fig. 3E). These results confirm induction of EMT in Cd exposed cells.

Cd exposure dysregulates miR-30 and SNAIL expression in lung epithelial BEP2D cells

Our results show that Cd exposure downregulated miR-30 family genes and upregulated SNAIL in BEAS-2B cells. We next asked whether Cd exposure could elicit similar response in other cell types. To answer this, we exposed lung epithelial BEP2D cells to several doses of Cd. Similar to our observations in BEAS-2B cells, we found dose-dependent downregulation of miR-30 family miRNAs in Cd exposed BEP2D cells (Fig. 4A). Furthermore, we found dose-dependent increase in the SNAIL mRNA (Fig. 4B) and protein

levels (Fig. 4C). These results suggest that Cd exposure-induced repression of miR-30 family genes and upregulation of SNAIL is not a cell-type specific phenomenon.

Cd induces SNAIL expression through miR-30 downregulation

miR-30 family miRNAs are known to negatively regulate SNAIL by targeting its 3' UTR (Kumarswamy et al., 2012; Zhang et al., 2012; Yang et al., 2017). Therefore, it is conceivable that SNAIL upregulation in Cd exposed cells could be a consequence of miR-30 downregulation. Therefore, we next examined whether miR-30 directly targets SNAIL 3' UTR in BEAS-2B cells. Since miR-30e was the most downregulated among the miR-30 family members in Cd exposed cells (Fig. 2D), we generated a luciferase reporter construct by inserting wild-type SNAIL 3' UTR containing miR-30e target site downstream of luciferase coding sequence in pIS0 vector (WT-SNAIL) (Fig. 5A). As control, we used a SNAIL 3' UTR luciferase construct in which the miR-30e target site was mutated (mut-SNAIL) (Fig. 5B). The WT and mutant constructs were stably transfected into BEAS-2B cells and the cells were treated with Cd for 24 h. We found higher luciferase expression in the WT-SNAIL cells treated with Cd, compared to untreated cells (Fig. 5C). On the contrary, the luciferase expression remained the same in the mut-SNAIL cells with or without Cd exposure (Fig. 5C). This suggests that miR-30e directly targets SNAIL 3' UTR causing its repression.

We next wanted to examine if increase in the levels of miR-30 could prevent Cd-induced SNAIL upregulation. To accomplish this, we transfected miR-30e mimic or miRNA mimic-negative control (mimic-NC) into WT-SNAIL and mut-SNAIL BEAS-2B cells. The cells were then exposed to Cd. miR-30e mimic transfected WT-SNAIL cells showed significantly reduced luciferase expression, compared to cells transfected with mimic-NC (Fig. 5D). On the other hand, no change in luciferase expression was detected in the mut-SNAIL cells transfected with miR30e mimic or mimic-NC. These results suggest that miR-30e mimic could prevent SNAIL upregulation in Cd exposed cells.

Next, we stably overexpressed miR-30e in BEAS-2B cells. As control, we used miRNA scrambled negative control plasmid. We then exposed the miR-30e-overexpressing and the miR-control BEAS-2B cells to 10 μ M CdCl₂ for 12 or 24 h. First, we confirmed overexpression of miR-30e in Cd treated cells by quantitating its miRNA levels (Fig. 5E). We then examined the levels of SNAIL in miR-control and miR-30e overexpressing cells. As expected, Cd exposure caused an increase in SNAIL mRNA (Fig. 5F) and protein levels (Fig. 5G) in the miR-control cells. In the miR-30e overexpressing cells, we found a robust decrease in SNAIL mRNA (Fig. 5F) and protein levels (Fig. 5G), suggesting that miR-30e overexpression abrogated Cd induced upregulation of SNAIL. These results confirm that Cd upregulates SNAIL through the downregulation of its negative regulator, miR-30.

Discussion

Environmental and occupational exposure to Cd is associated with several diseases including cancers (Wang et al., 2012; Nawrot et al., 2015). However, the mechanisms underlying the pathogenic potential of Cd remain unclear. In this study, we sought to identify the mechanisms underlying Cd exposure-induced lung diseases by examining the transcriptome

of Cd exposed lung epithelial cells. Our results show that Cd exposure upregulates SNAIL via downregulation of its repressors, miR-30 family miRNAs. miR-30e mimics and miR-30e overexpression prevented Cd-induced SNAIL upregulation. SNAIL expression is associated with a number of diseases associated with Cd exposure including cancer and metastasis. Therefore, our results suggest downregulation of miR-30 as an important mechanism underlying the lung diseases associated with Cd exposure.

miRNAs are small, 18–25 nucleotide-long, non-coding RNA molecules. miRNAs bind target mRNA molecule at the 3' UTR and silence genes through mRNA degradation or translation inhibition (Macfarlane and Murphy, 2010). Our computational analysis of the genes that were differentially expressed by Cd exposure predicted 50% of the upregulated genes to be miRNA targets. Recent studies have highlighted the impact of Cd exposure on the expression of miRNAs in humans, animals and cells. Extensive changes in miRNA expression profile have been identified in the renal cortex of Cd exposed rats. Consequently, miRNAs have been proposed as biomarkers of Cd induced kidney injury (Fay et al., 2018). Furthermore, Cd exposure altered the expression of several miRNAs in human placenta and placental trophoblasts (Brooks et al., 2016; Brooks and Fry, 2017). This suggests that deregulation of miRNA-based mechanisms could be important in Cd pathogenesis.

Notably, a quarter of the potential miRNA target genes that we identified were targets of miR-30 family members (Fig. 2C). The miR-30 family contains six mature miRNAs, miR-30a, miR-30b, miR-30c-1, miR-30c-2, miR-30d, and miR-30e (Yang et al., 2017; Mao et al., 2018). miR-30 family is frequently found to be downregulated in cancers of lung, colon, prostate, thyroid, breast and liver and in leukemia (Yang et al., 2017; Croset et al., 2018; Mao et al., 2018). Due to this reason they have been classified as tumor suppressors (Croset et al., 2018; Mao et al., 2018). Our analysis showed that all the members of the miR-30 family were downregulated in Cd exposed lung epithelial BEAS-2B and BEP2D cells (Fig. 2D, 4A). Interestingly, we identified the transcription factor, SNAIL as the most upregulated among the potential miR-30 targets. The most recognized regulatory function of SNAIL is its ability to induce epithelial-mesenchymal transition (EMT) (Lamouille et al., 2014). During EMT, the epithelial cells lose their cell-cell adhesion structures such as adherens junctions and lose epithelial cell polarity, and acquire invasive and migratory mesenchymal phenotype (Le Bras et al., 2012; Lamouille et al., 2014). EMT is a key process in embryonic development and wound healing and the role of EMT in lung development is well established (Kalluri and Weinberg, 2009). However, EMT is also a pathogenic process associated with a number of diseases including cancer and metastasis (Kalluri and Weinberg, 2009; Jolly et al., 2018). SNAIL, a major driver of EMT, is a negative regulator of several epithelial proteins including E-Cadherin (CDH1). CDH1 is a mediator of cell adhesion, which is mainly expressed in the epithelial cells (Kalluri and Weinberg, 2009). SNAIL binds to the three E-box sequences in the CDH1 promoter and directly represses its transcription (Battle et al., 2000). Reduction in the levels of CDH1 lowers cell-cell contacts and increases migration and invasion potential of the cells. Therefore, loss of CDH1 expression is considered a hallmark of epithelial-mesenchymal transition (EMT). In addition to CDH1, SNAIL also inhibits the tight junction proteins important for epithelial cell polarity, claudin and occludin (Ikenouchi et al., 2003). Furthermore, SNAIL is an upstream activator of ZEB1, another mesenchymal protein, whose increased expression is identified as a driver of EMT.

Due to these reasons SNAIL is considered an EMT master regulator. Our results show that SNAIL upregulation in Cd exposed cells is associated with characteristics of mesenchymal phenotype including CDH1 downregulation, and ZEB1 and vimentin upregulation. These results suggest induction of EMT in Cd exposed cells.

miR-30 is a negative regulator of SNAIL (Kumarswamy et al., 2012; Zhang et al., 2017). Our results (Fig. 5C) as well as earlier studies (Kumarswamy et al., 2012; Zhang et al., 2012; Zhang et al., 2017) show that miR-30 could repress SNAIL expression by directly targeting its 3' UTR. Therefore, it is conceivable that Cd-induced downregulation of miR-30 could relieve the miR-30-dependent repression of SNAIL resulting in increased SNAIL protein levels and acquisition of EMT phenotype. Confirming this, overexpression of miR-30e and transfection of miR-30e mimics reversed the effects of Cd on SNAIL expression (Fig. 5D, F).

Cd is a carcinogenic metal associated with increased risk of lung cancer and metastasis in humans. In addition, chronic human lung and airway diseases including pulmonary fibrosis and chronic obstructive pulmonary disease (COPD) are major deleterious outcomes of Cd exposure. Interestingly, EMT is a major driver of Cd exposure-induced lung diseases (Jolly et al., 2018). Therefore, Cd induced downregulation of miR-30, and the consequential SNAIL upregulation could be an important mechanism underlying the lung diseases associated with Cd exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A number of genes upregulated by Cd exposure are targets of miR-30 family miRNAs
- miR-30 family is downregulated in Cd exposed BEAS-2B and BEP2D cells
- Cd induced miR-30 repression caused upregulation of the EMT master regulator, SNAIL
- miR-30e overexpression and miR30e mimics prevented Cd-induced SNAIL upregulation.

Figure 1. Cadmium exposure affects cancer associated biological processes in BEAS-2B cells.

A. BEAS-2B cells were treated with 5 μM CdCl₂ for 72 h and gene expression was examined using RNA-Seq analysis. RNA-Seq results are displayed as volcano plot. The differentially expressed genes are shown in red (fold change ≥ 1.5; padj < 0.05). B. The top enriched GO biological processes in Cd exposed cells. (C and D). qPCR analysis showing dose-dependent changes in the expression levels of candidate upregulated (C) and down-regulated (D) genes. Statistical significance was evaluated using *t*-test ($P < 0.05^*$; $P < 0.01^{**}$; $P < 0.001^{***}$; $P < 0.0001^{****}$).

Figure 2. Genes upregulated by Cd exposure in BEAS-2B cells are enriched for miRNA targets.

A. miRNA target enrichment analysis (ToppFun and target scan analysis) in the up- and down-regulated gene sets. While 50% of the upregulated genes were predicted to be targets of miRNAs, no downregulated genes were predicted as miRNA targets (q-value FDR $B \& Y < 0.05$). B. miRNA binding site enrichment analysis (Target scan) in the up-regulated gene set showing the top 10 enriched miRNAs (q-value FDR $B \& Y < 0.05$). C. The upregulated genes are enriched for miR-30 targets. (ToppFun and target scan analysis). D. qPCR analysis showing dose-dependent downregulation of miR-30 family miRNAs in Cd exposed cells. The results are shown as fold change compared to untreated cells. *SNORD44* was used as internal control. Error bars correspond to the standard deviation of at least two biological replicates. Statistical significance was evaluated using *t*-test ($P < 0.05^*$; $P < 0.01^{**}$; $P < 0.001^{***}$).

Figure 3. SNAIL is upregulated in Cd-exposed BEAS-2B cells.

A. Top upregulated miR-30 target genes in Cd exposed cells. B. qPCR analysis showing dose-dependent upregulation of *SNAI1* in BEAS-2B cells exposed to various doses of CdCl₂ for 72 h. GAPDH was used as internal control. Error bars correspond to the standard deviation of at least two biological replicates. Statistical significance was evaluated using *t*-test ($P < 0.01^{**}$). C. Representative Western blotting images and quantification of the band intensities. Dose-dependent upregulation of SNAIL occurs in BEAS-2B cells exposed to CdCl₂ for 72 h. The ratio of SNAIL to α -tubulin (loading control) is estimated and the band intensities are displayed as fold change over control. Each column represents the mean of 2 biological replicates. D. Representative Western blotting images and quantification of the band intensities. Time-dependent upregulation of SNAIL occurs in BEAS-2B cells exposed to 10 μ M CdCl₂. The ratio of SNAIL to α -tubulin (loading control) is estimated and the band intensities are displayed as fold change over control. Each column represents the mean of 2 biological replicates. E. Representative Western blotting images and quantification of the band intensities. Downregulation of epithelial marker CDH1, and upregulation of mesenchymal markers ZEB1 and vimentin occurs in BEAS-2B cells exposed to 10 μ M CdCl₂ for 72 h. The ratios of CDH1, ZEB1 and vimentin to α -tubulin (loading control) are estimated and the band intensities are displayed as fold change over control. Each column represents the mean of 2 biological replicates. Statistical significance of western blotting results was evaluated using *t*-test ($P < 0.05^{*}$; $P < 0.01^{**}$; $P < 0.001^{***}$).

Figure 4. Cd exposed BEP2D cells show downregulation of miR-30 family miRNAs and upregulation of SNAIL.

A. qPCR analysis showing dose-dependent downregulation of miR-30 family miRNAs in Cd exposed BEP2D cells. The results are shown as fold change compared to untreated cells. *SNORD44* was used as internal control. B. qPCR analysis showing dose-dependent upregulation of *SNAIL* in Cd-exposed BEP2D cells. *GAPDH* was used as internal control. Error bars (A and B) correspond to the standard deviation of at least two biological replicates. Statistical significance was evaluated using *t*-test ($P < 0.05^*$; $P < 0.01^{**}$; $P < 0.001^{***}$). C. Representative Western blotting images and quantification of the band intensities. SNAIL is upregulated in a dose-dependent manner in BEP2D cells exposed to CdCl_2 for 72 h. The ratio of SNAIL to α -tubulin (loading control) is estimated and the band intensities are displayed as fold change over control. Each column represents the mean of 2 biological replicates. Statistical significance of western blotting results was evaluated using *t*-test ($P < 0.05^*$; $P < 0.001^{***}$).

Figure 5. Cd exposure upregulates SNAIL through miR-30 repression.

A. Predicted miR-30e binding site at the SNAIL 3' UTR (TargetScan). For luciferase assay, SNAIL 3' UTR containing wild-type miR-30e binding site was cloned into the luciferase reporter vector (wild-type SNAIL). B. SNAIL 3' UTR of containing mutated miR-30e binding site. Mutated bases are underlined. For luciferase assay, SNAIL 3' UTR containing mutant miR-30e binding site was cloned into the luciferase reporter vector (mutant SNAIL). C. Luciferase assay in BEAS-2B cells stably expressing wild-type SNAIL (WT-SNAIL) or mutant SNAIL (mut-SNAIL) 3' UTR luciferase reporter constructs. Cd exposure caused increased luciferase expression in the WT-SNAIL cells, but not in the mut-SNAIL cells, suggesting that miR-30e directly targeted SNAIL 3' UTR. The results are shown as fold change compared to untreated cells. D. Luciferase assay in BEAS-2B cells stably expressing wild-type SNAIL (WT-SNAIL) or mutant SNAIL (mut-SNAIL) 3' UTR luciferase reporter constructs transfected with miR-30e-mimic or miR mimic negative control (mimic-NC). Upon transfection with miR-30-e mimic, WT-SNAIL cells (containing wild-type miR30-e binding sequence) showed decrease in luciferase expression. However, mut-SNAIL cells (containing mutant miR30-e binding sequence) did not show any change in luciferase expression. The results are shown as fold change compared to cells transfected with mimic-NC. E. qPCR analysis showing miR-30e levels in Cd-exposed BEAS-2B cells stably overexpressing miR-30e or miR-control. F. qPCR analysis showing *SNAIL* mRNA levels in Cd-exposed BEAS-B cells stably overexpressing miR-30e or miR-control. Error bars (C-F) correspond to the standard deviation of at least two biological replicates. Statistical significance was evaluated using *t*-test ($P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***). ns: not significant G. Representative Western blotting images and quantification of the band intensities. Stable overexpression of miR-30e reduced SNAIL upregulation in BEAS-B cells exposed to Cd. Statistical significance of western blotting results was evaluated using *t*-test ($P < 0.01$ **).