Oncogene (2005) 24, 4873–4882

www.nature.com/onc

ORIGINAL PAPERS

The Human mineral dust-induced gene, *mdig*, is a cell growth regulating gene associated with lung cancer

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Environmental or occupational exposure to mineral dusts, mainly silica and asbestos, is associated with an increased incidence of lung inflammation, fibrosis, and/or cancer. To better understand the molecular events associated with these pulmonary diseases, we attempted to identify genes that are regulated by mineral dusts. Using a differential display reverse transcription polymerase chain reaction technique and mRNAs of alveolar macrophages from both normal individuals and coal miners, we identified a novel mineral dust-induced gene named *mdig*, which had not been fully characterized. The expression of *mdig* mRNA was detected in alveolar macrophages from coal miners but not from normal subjects. The inducible expression of *mdig* could be observed in A549 cells exposed to silica particles in a time-dependent manner. The full-length *mdig* mRNA was expressed in human lung cancer tissues but was barely detectable in the adjacent normal tissues. In addition, a number of lung cancer cell lines constitutively express *mdig*. Alternative spliced transcripts of *mdig* were detected in some lung cancer cell lines. Silencing *mdig* mRNA expression in A549 lung cancer cells by siRNA-mediated RNA interference inhibits cell proliferation and sensitizes the cells to silica-induced cytotoxicity. These results suggest that the *mdig* gene may be involved in the regulation of cell growth and possibly the development of cancer.
Oncogene (2005) **24,** 4873–4882. doi:10.1038/sj.onc.1208668; published online 9 May 2005

Keywords: mineral dust; *mdig*; lung cancer

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The cDNA sequences reported in this paper have been deposited in the GenBank with access numbers: BE441202, AY302110 and AY456380.
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Received 29 November 2004; revised 22 February 2005; accepted 23 February 2005; published online 9 May 2005

Introduction

Inhalation of airborne mineral dust is a costly occupational hazard, both in terms of the quality of life for the worker and national expenditure for lost work production, health care and disability compensation (Mossman *et al*., 1990; Rom *et al*., 1991; Kuempel *et al*., 2001; Nelson and Kelsey, 2002). Tumorigenic and/or fibrotic lung diseases that result from the mining, milling, processing, and use of mineral dusts severely compromise pulmonary function and may lead to lung cancer or death in those workers who encounter mineral particulate matter on a chronic basis (Korn *et al*., 1987). Dust-induced lung diseases, particularly those caused by exposure to asbestos and silica, have been linked to occupational lung cancer, and continue to produce a significant drain on human health and governmental resources in the future, because of the prolonged and progressive nature of these diseases (asbestosis and silicosis continue to develop even after exposure ceases) and because of the failure of industry to comply with the permissible or recommended exposure limits in certain work environments (CDC/NIOSH, 2003). However, the effect of various dust levels on biological systems is still an enigma. Thus, the etiology and molecular mechanisms of lung diseases that result from the interaction of dust particles with cellular components of the lung deserve careful and detailed investigation.

The alveolar macrophage (AM) appears to be the most important cell type involved with the progression of mineral dust-induced lung diseases (Brody *et al*., 1982). The AM not only functions as the primary cell type that interacts with inhaled particulate matter, but possesses a formidable arsenal of lipid and peptide mediators, which can both stimulate and attenuate the defensive capacity of the lung (Ortiz *et al*., 2001). AMs activated by soluble and particulate stimuli secrete more than 100 potential mediators to orchestrate a complex pattern of intercellular communication among macrophages, fibroblasts, epithelial cells, and circulating immune cells. It is through the synthesis and release of these substances that the AM manifests its control on the biological responses of other lung cell types. These biological responses include cell proliferation, cell cycle transition, cell apoptosis, gene expression, and/or

transformation. Among these substances are peptidal growth factors, cytokines, proteolytic enzymes, and oxygenated derivatives of arachidonic acid (Ortiz *et al*., 1999). It is becoming apparent that the uncontrolled elaboration of AM secretory products, as a result of chronic inhalation of particulate matter, may overwhelm biological defense mechanisms and thus alter the metabolic milieu in the lung in favor of a biochemical environment that promotes the pathogenesis of lung disease.

The most prominent products released from AMs in response to mineral dust exposure are the fibrotic and/or inflammatory cytokines, including IL-1, IL-6, IL-8, IL-12, TNFα, and several other factors. However, expression of cytokine genes induced by mineral dust exposure could not convincingly explain the progressive nature of the fibrotic and the carcinogenic effects of mineral dust on lung cells. Therefore, we entertained the possibility that there must be additional novel gene(s) regulated by this pathway that contribute to the harmful effects of mineral dust.

In the present study, we report the identification of a mineral dust-induced gene (*mdig*, GenBank Access number: BE441202; AY302110; AY456380) through the use of a differential display reverse transcription–polymerase chain reaction (DDRT–PCR), using mRNAs extracted from AMs from a human nonminer control population and from subjects who are actively working in coal mines. Search of the GenBank database revealed no homology with any of the known genes during the first deposition of the cDNA sequence into the gene bank. Further study demonstrated that the mRNA of *mdig* was highly expressed in AMs from miners and in a number of lung cancer samples and lung cancer cell lines. Furthermore, an alternative mRNA splicing of *mdig* in some lung tumor cell lines has been demonstrated.

Results

Differences in mRNA expression in AMs obtained from control subjects and coal miners

This experiment involved collection of AMs from both normal individuals and from coal miners by bronchoalveolar lavage (BAL) as performed under institutional approved procedures. AMs from three normal individuals and three coal miners were pooled together, respectively. Total RNA was extracted from the AMs and subjected to DDRT–PCR analysis. DDRT–PCR has been shown to be a suitable approach for identifying differentially regulated genes in a variety of cells. To study changes in gene expression, total RNA was used in DDRT–PCR with a set of arbitrary/anchored DDRT–PCR primers and [^{33}P]dCTP to detect differentially displayed mRNAs in AMs from miners (M) and normal individuals (N). The products of the DDRT–PCR were separated on a denaturing polyacrylamide gel, and normal (N) lanes *versus* miner (M) lanes were compared to identify bands of different intensity (Figure 1a). Visual inspection of resultant autoradiograms revealed that the majority of bands exhibited a similar intensity between N and M lanes, using various PCR primer sets. However, subtle differences were noted. Several mRNA bands were uniquely expressed only in AMs from miners, such as those circled in Figure 1a.

To determine the nature of these highly expressed mRNAs in miner AMs, three prominent bands (circled in Figure 1a) in M lane were randomly selected and excised from the gel and reamplified. The reamplified cDNA was cloned into a pCR-TRAP vector, and the cDNA inserts were sequenced in both directions. Analysis of the obtained DNA sequence data indicated that all clones had flanking primer sequences identical to those used in the original DDRT–PCR. One of these cloned products, clone 2, gave an exact sequence match to human integrin αE in a BLAST search (Figure 1b, left panel). The remaining two clones, clone 3 (Figure 1b, right panel) and clone 6 (data not shown), showed no homology with any known genes in the Genbank, indicating that these two mRNAs might be products of unidentified novel genes. By searching the expression sequence tag (EST) database, several EST fragments that exhibited significant homology with clone 3 were identified. The majority of these clone 3 homology EST fragments were derived from lung cancer, melanoma, parathyroid tumor, ovary tumor, colon tumor, or breast cancer, suggesting that clone 3 might be produced by a tumor-related gene. No homology EST sequences for clone 6 were found in the EST database.

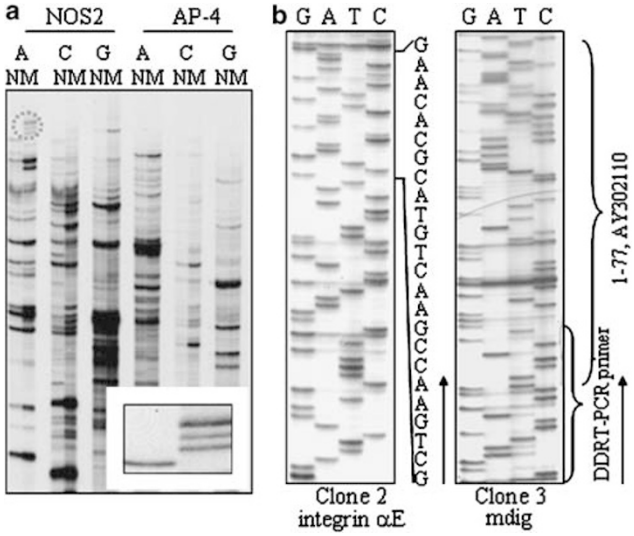

Figure 1 Difference in mRNA expression in AMs from normal individuals and coal miners. (**a**) DDRT–PCR of total RNA from AMs pooled from three normal individuals (N) and three miners (M). A, C and G denote arbitrary primers; the anchored primer NOS2 was derived from human iNOS gene in the region of 2884–2904 (GenBank ID U20141); the anchored primer AP-4 was purchased from GenHunter Co. (Nashville, TN, USA). The circle indicates clone 2, clone 3, and clone 6 expressed only in AMs from miners. The insert shows the circled three bands expressed in miner AMs. (**b**) DNA sequencing of clone 2 and clone 3

Clone 3 encodes a protein with 465 amino acids

To determine the characteristics of the novel gene, clone 3, successive sequencing steps were used to assemble the full-length sequence of the cDNA fragment inserted in the pCR-TRAP vector. The coding region of the nucleotide sequence and corresponding amino acids are shown in Figure 2. The predicted ATG start code for methionine is located 117 bp downstream from the 5′ end of the cDNA fragment and is included in a single open reading frame of 465 amino acids with a predicted molecular weight of 52 800.2 kDa and pI 6.23. A translational TAG stop code is located 1512 bp downstream from the 5′ end. Since the mRNA of clone 3 was derived from AMs of miners who were repeatedly and chronically exposed to mineral dust, we originally named this novel gene as *mdig*. The predicted amino-acid sequence of Mdig contains several putative phosphorylation sites for protein kinases and a JmjC domain at the amino terminus followed by a cupin domain and an MarR domain (data not shown). Structural prediction suggests that the Mdig protein contains a number of helix–loop–helix domains and two coiled-coil structures at positions 120–160 and 310–340 (data not shown). Reinhardt's analysis indicates that the Mdig protein is a nuclear protein.

Tissue distribution of mdig *mRNA*

To determine the tissue distribution of *mdig* mRNA, Northern blotting analysis was performed by using the human eight-lane MTN blot and a *mdig*-specific probe under stringent hybridization and washing conditions. The MTN blot contains mRNAs from eight different normal human tissues. The results of this experiment revealed a limited tissue expression of *mdig* mRNA. The *mdig* mRNA were detected in liver, skeletal muscle, heart, pancreas, and placenta (Figure 3). In the normal tissues from brain, lung, and kidney, the expression of *mdig* was undetectable. The length of the detected mRNA species was 1.5 kb, possibly representing the fully spliced *mdig* mRNA, and 0.4–0.5 kb, probably corresponding to an alternative-spliced mRNA.

Identification of the exon–intron structure of the mdig *gene*

Based on the available human genome data in the GenBank, we used a BLAST sequence analysis program and characterized the human *mdig* exon–intron organization together with the potential promoter regions. Our analysis indicates that the *mdig* gene is located in human chromosome 3. It contains 10 exons and spans about 30 kb. Table 1 showed the sizes of individual exons and introns, and the sequences immediately flanking the exon–intron junctions. All of the intron sequences at these junctions follow the *gt-ag* rule. The first exon and the 5′ −40 nt of exon 2 represent the 5′-untranslated region of the *mdig* mRNA. Introns 5 and 7 interrupt the coding region of the gene at codon 262 for Asn and 354 for Gly, respectively.

Increased expression of mdig *in AMs from miners or silica-treated A549 cells*

To verify *mdig* is a mineral dust-inducible gene, we performed RT–PCR experiments using AMs collected from eight normal individuals and 11 miners who had a history of frequent mineral dust exposure. The results depicted in Figure 4a indicate that the expression of *mdig* was undetectable in the AMs from all eight normal individuals. In contrast, *mdig* was highly expressed in the AMs from four of the 11 miners, although with different levels of intensity.

Owing to the difficulty of obtaining more samples of human AM for further detailed studies, we next used lung cancer cell lines to characterize the biochemical feature of *mdig* gene. In A549 cells, we noted an appreciable induction of *mdig* in the cells treated with 100 μg/ml of silica for 18 h (Figure 4b). Semiquantitative RT–PCR experiments suggested a 2.5-fold induction of *mdig* mRNA by silica treatment for 18 h (Figure 4b, left panel). Treatment of the cells with various concentrations of silica for 18 h indicated that the induction of *mdig* by silica is also dose-dependent with peak of 100–200 μg/ml of silica (Figure 4c).

Human lung cancer samples and lung cancer cell lines express mdig

To determine whether *mdig* is expressed in cancer tissues or cells, we initially examined seven human lung cancer samples for the expression of *mdig* by RT–PCR. Most of these lung cancer samples showed expression of *mdig* (Figure 5a, lanes 6–12). In contrast, the expression of *mdig* was barely detectable in the noncancerous surrounding tissues corresponding to the malignant tissues of samples 6–10, except the normal tissue (Figure 6a, lane 5) adjacent to mesothelioma (sample 10). We next screened 19 human lung cancer cell lines by RT–PCR with primers that encompass the whole open reading frame of the *mdig* gene with a length of 1501 bp. The data showed that 15 cell lines expressed *mdig* under basal conditions (Figure 5b). In addition to the expected 1.5 kb PCR-amplified fragment, two cancer cell lines, H441 and H2347, also expressed a short version of *mdig* (Figure 5b), suggesting possible alternative splicing of *mdig* mRNA in some cancer cell lines.

Alternative splicing of mdig *mRNA in H441 cells*

The alternative transcript of a given mRNA is produced as a result of either the use of alternative promoter(s) or the involvement of alternative mRNA splicing. To address this issue, we employed several sets of PCR primers that encompass different exon regions (Figure 6a). Figure 6b shows that all of the PCR primer sets could amplify *mdig* mRNA in A549 cells with the expected sizes. In H441 cells, the main fragments amplified by primer sets I and III are about 1250 and 350 bp, respectively, whereas the fragments with expected sizes of 1510 and 823 bp as seen in A549 cells are very weakly amplified (Figure 6b).

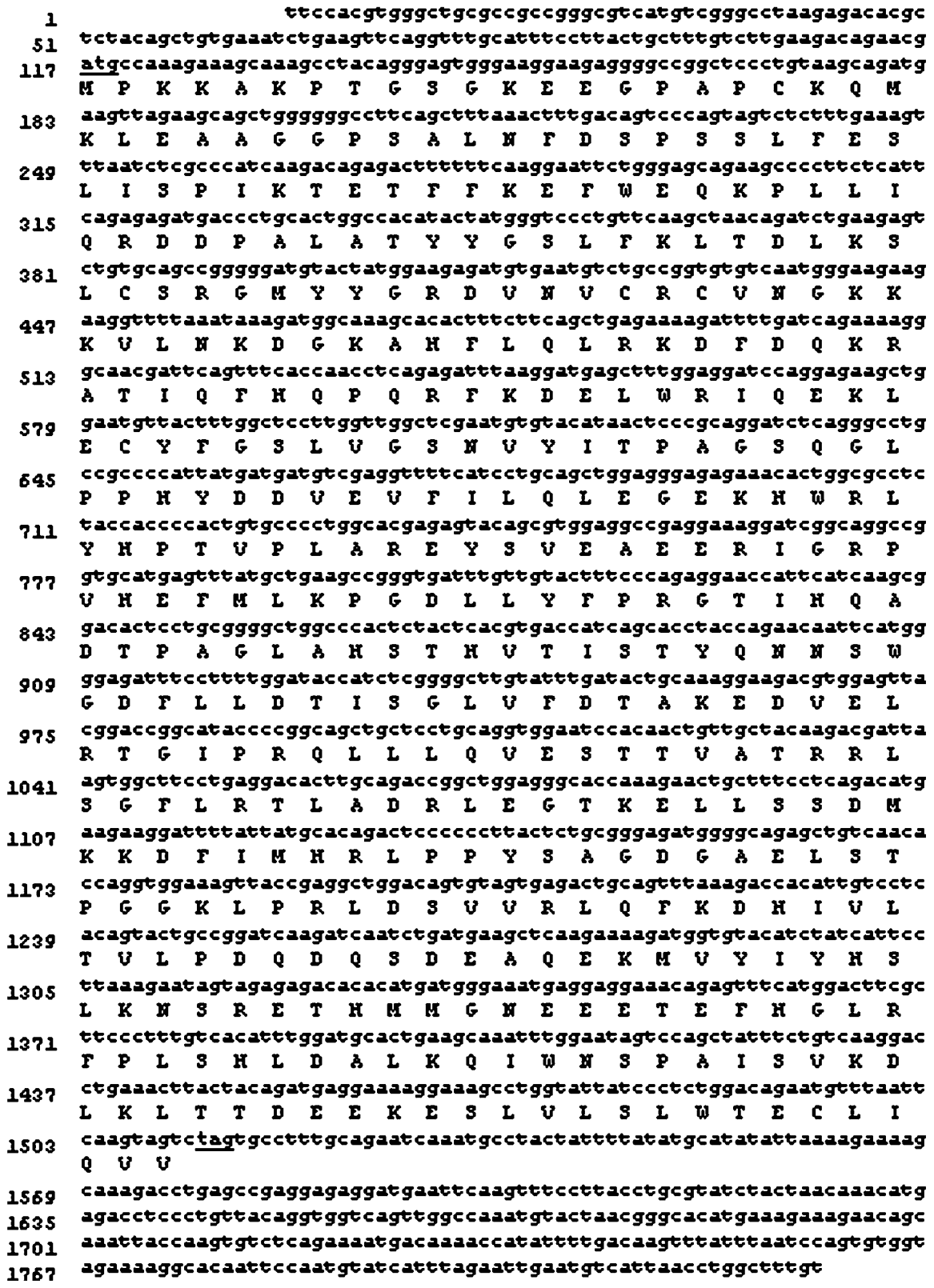

Figure 2 DNA sequence and protein coding region of *mdig* gene. The translation start code ATG and stop code TAG have been underlined. The extended 3′-part of *mdig* cDNA (523 bp) is not shown (this sequence has been deposited in GenBank with access number AY302110)

To determine the nature of alternative splicing of *mdig* in H441 cells, the RT–PCR products amplified using primer sets I and III were purified and sequenced (Figure 6c). Analysis of the DNA sequence data suggests that the principal *mdig* mRNA in H441 cells, *mdig2* (GenBank Access number: AY456380), lacks the entire region of exon 2 (471 bp) but contains a 207 bp region that represents a new alternative exon inserted between exon 5 and exon 6 (Figure 6c and d). Thus, the sizes of the principal RT–PCR-amplified fragments in H441 cells, using primer sets, I and III, should be 1246 and 353 bp, respectively. The fragment sizes measured in the agarose gel confirmed this supposition (Figure 6b, left panel). Owing to the alternative splicing that skipped the entire exon 2 region, the ATG starter code for Mdig2 is located in the inserted exon which generates seven new amino acids. The remaining amino-acid sequence is identical with the C-terminal 203 amino-acid sequence of the Mdig protein. The new protein lacks the JmjC homology domain. The usage of exons of *mdig* mRNA from A549 and H441 cells is schematically illustrated in Figure 6d.

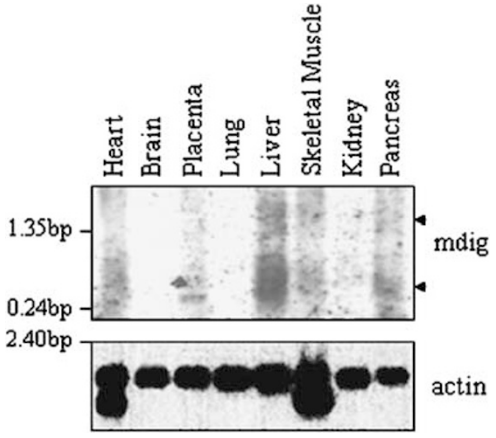

Figure 3 Mdig mRNA is expressed in several normal human tissues. Northern blotting was performed using human eight-lane MTN blot and cDNA probes for *mdig* (upper panel) and *β*-actin (lower panel)

Involvement of Mdig protein in cell growth

In order to investigate the function of the *mdig* gene, we designed specific siRNAs against human *mdig* and transfected these siRNAs into A549 cells. The *mdig* siRNAs effectively reduced the level of *mdig* mRNA after 12 or 24 h of transfection, based on RT–PCR analysis of *mdig* expression in A549 cells (Figure 7a). The control siRNA showed no effect on the level of *mdig* mRNA at neither 12 nor 24 h (data not shown). Several parameters for cell growth were determined after the transfection of the *mdig* siRNA. A time-course study indicated that transfection of the cells with both *mdig* siRNAs showed a significant inhibition of cell proliferation compared to the control siRNA against luciferase (Figure 7b). Figure 7c indicates a substantial delay in the

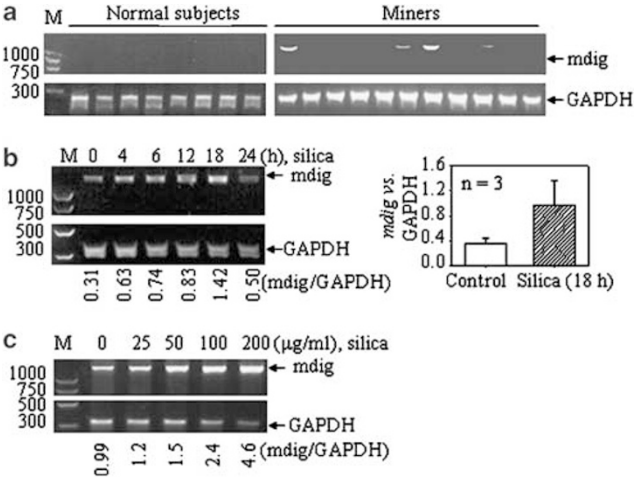

Figure 4 Increased expression of *mdig* in coal miner AMs and silica-induced lung cancer cells. (**a**) RT–PCR analysis of *mdig* mRNA expression in AMs from eight normal individuals and 11 miners. (**b**) Induction of *mdig* by 100 *μ*g/ml of silica in A549 cells. The numbers at the bottom of this panel show ratio of *mdig* mRNA *versus* GAPDH mRNA quantified by densitometry. The left panel shows the result of three semiquantitative RT–PCR using total RNA from A549 cells cultured in the absence or presence of 100 *μ*g/ml of silica for 18 h. (**c**) Dose-dependency of silica-induced *mdig* expression. The cells were treated with the indicated concentrations of silica for 18 h. The numbers at the bottom of this panel show ratio of *mdig* mRNA *versus* GAPDH mRNA quantified by densitometry

Table 1 Sizes of individual exons and introns of the *mdig* gene

Exon number	*Exon size (bp)*	*Sequence at exon-intron junction*		*Codon interrupted*	*Intron size (bp)*
		5′ Splice donor	*3′ Splice acceptor*		
1	>100	TGAAGTTCAGgtgtgtgggg	ctttggtaagGTTTGCATTT		426l
2	471	GAGATTTAAGgtaaccagtt	ttttgtaaagGATGAGCTTT		5453
3	120	TGATGTCGAGgt aagagatg	gggtctctagGTTTTCATCC		2409
4	129	TATGCTGAAGgtatgtgata	cttcctttagCCGGGTGATT		4555
5	104	ACCAGAACAAgtaaacactg	catatcctagTTCATGGGGA		3503
6	103	GCTGCTCCTGgtiiggigti	gtgttcctagCAGGTGGAAT	Asn^{252}	770
7	172	TCAACACCAGgtggagcctg	ggttttctagGTGGAAAGTT		2394
8	89	AGATCAATCTgtgagtatcc	ttccccaaagGATGAAGCTC	Gly^{334}	1473
9	90	GGAAACAGAGgtttgtttca	atcctttcagTTTCATGGAC		455
10	>400				

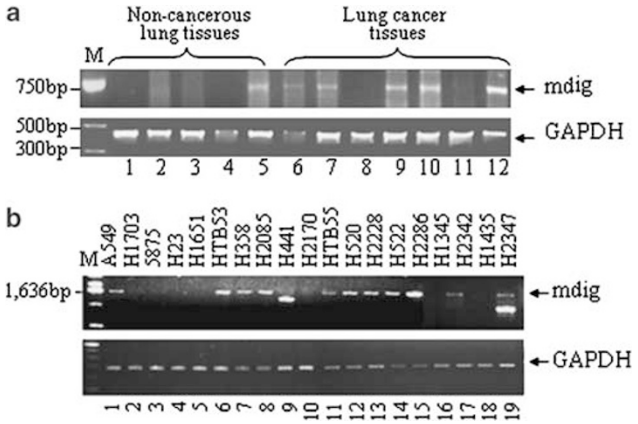

Figure 5 Expression of *mdig* in human lung cancer tissues and lung cancer cell lines. (**a**) The expression of *mdig* was compared between malignant tissues (lanes 6–12) and their corresponding noncancerous surrounding tissues (lanes 1–5). The noncancerous surrounding tissues for samples 11 and 12 were not available. (**b**) Expression of *mdig* in lung cancer cell lines

formation of cell confluence after 24 h of *mdig* mRNA transfection, another measure supporting the observation that silencing *mdig* mRNA dampens cell growth. There is no appreciable alteration in cell morphology (Figure 7d) in the cells treated with *mdig* siRNA and cultured in normal medium.

Analysis of the cell cycle profile indicated that silencing of *mdig* mRNA delayed the cell cycle transition from G1 phase to S phase (Figure 8a), which likely explains observed inhibition of cell proliferation (Figure 7b and c). To determine the effect of *mdig* siRNA on mineral dust-induced cellular response, the A549 cells were cultured in the low serum medium (0.1% FBS) and treated with 100 μg/ml of silica particles for 18 h. Biochemical analysis of lactic dehydrogenase (LDH) activity, an index of cytotoxicity, revealed a similar basal level of LDH release from the cells transfected with the control and *mdig* siRNAs (Figure 8b, open bars). Silencing of the *mdig* mRNA by siRNA increased silica-induced LDH release substantially (Figure 8b,

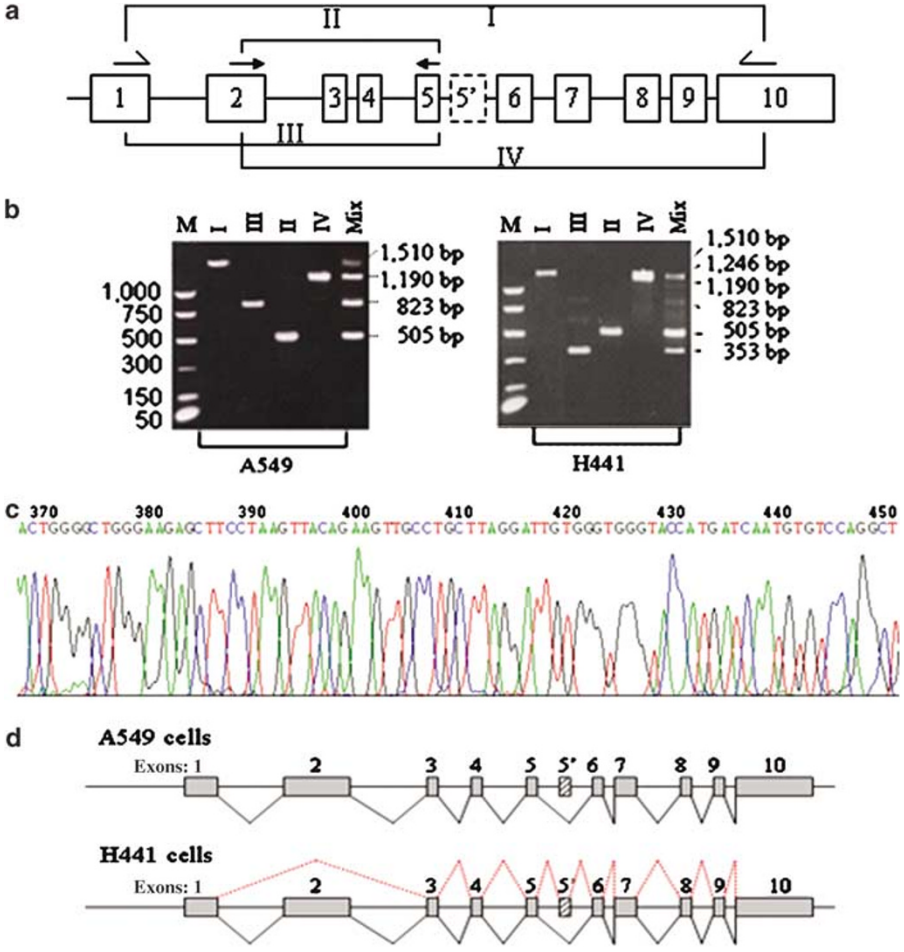

Figure 6 Alternative splicing of *mdig* mRNA. (**a**) Schematic demonstration of RT–PCR primers in correspondence to their exons in the *mdig* gene. (**b**) RT–PCR assay of *mdig* mRNA expression in A549 cells (left) and H441 cells (right). The PCR primer sets were indicated on the top of each panel. (**c**) DNA sequencing of primer extension showed a new region of *mdig* mRNA in H441 cells, which resulted from the use of an alternative exon (5′) with a size of 207 bp. Only the region of the first 80 nucleotides of this new exon is shown. (**d**) Schematic illustration of alternative transcripts produced by H441 cells (dashed line). 5′ denotes an alternative exon between exon 5 and exon 6

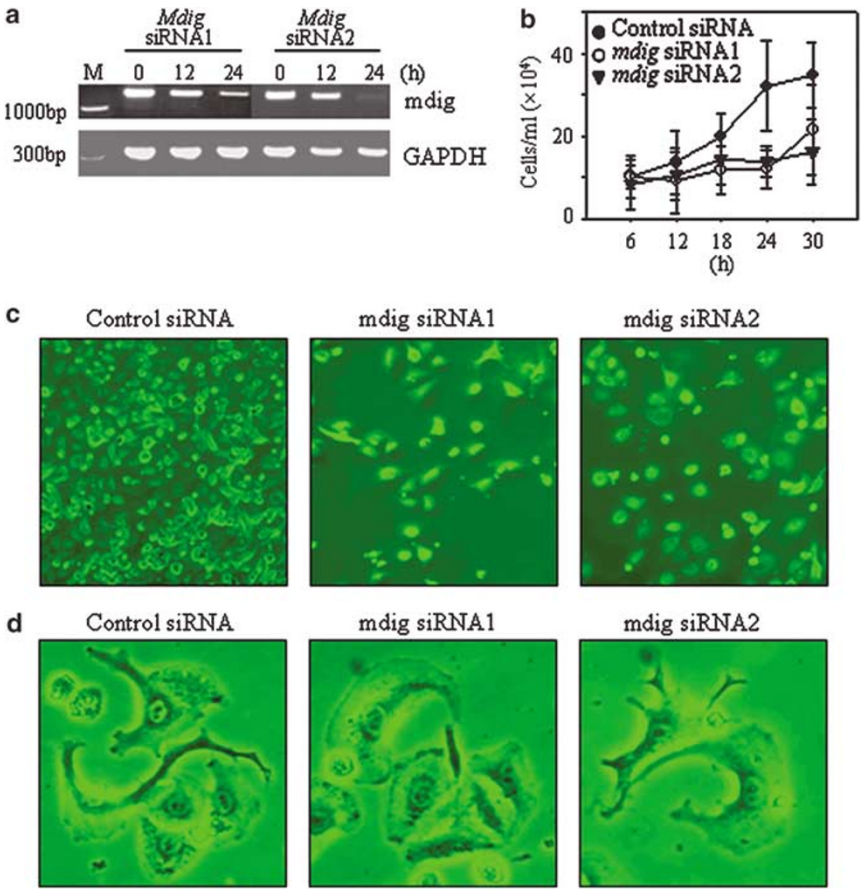

Figure 7 Cell growth regulation by *mdig*. (**a**) RT–PCR analysis of *mdig* mRNA in A549 cells transfected with siRNAs that target 1084–1104 region and 1221–1241 region of *mdig* mRNA, respectively, for 0, 12, or 24 h. (**b**) Time-course study of cell proliferation in the cells transfected with the indicated siRNA. Data are expressed as mean ± s.d. ($n = 6$). (**c**) Formation of cell confluence in A549 cells transfected with the indicated siRNAs. (**d**) Cell morphology assay of the cells transfected with the control siRNA or *mdig* siRNA

hatched bars), suggesting that knockdown of *mdig* mRNA sensitizes the cells for silica-induced cytotoxicity. Such effect was further confirmed by the MTT-based cytotoxicity analysis (data not shown).

Discussion

In this report, we have (i) identified a mineral dust-induced gene, *mdig*, by DDRT–PCR using mRNAs derived from AMs obtained from normal subjects and coal miners; (ii) demonstrated an increased expression of *mdig* mRNA in both AMs from miners and lung cancers; (iii) characterized the basic gene structure of the *mdig* and the tissue distribution of *mdig* mRNA; (iv) obtained strong evidence indicating an inducible expression of *mdig* mRNA by mineral dust and alternative transcripts of *mdig* in some tumor cell lines; and (v) revealed the involvement of *mdig* gene in cell growth and silica-induced cytotoxicity by an siRNA-mediated RNA interference technique.

The genes that are currently known to be upregulated by cellular exposure to mineral dusts include those associated with inflammatory and antiapoptotic responses. It is unknown whether there are other genes that can be regulated by mineral dusts. The *mdig* gene identified in this report is clearly a novel gene whose expression may be influenced by occupational exposure to mineral dusts. The function of the *mdig* gene has not been fully delineated. However, the presence of several conserved domains that are characteristic of chromatin remodeling and transcriptional regulation suggests that *mdig* may be a nuclear protein involved in gene transcription. Analysis of the encoded amino-acid sequence of *mdig* revealed a JmjC domain at the amino terminus followed by a cupin domain and an MarR domain. Proteins containing the JmjC domain have been implicated in eucaryotic and bacterial transcriptional regulation and chromatin remodeling (Clissold and Ponting, 2001), whereas proteins with the MarR domain are frequently found in some procaryotic RNA polymerases (Martin *et al*., 1996). In addition, the sequence and secondary structure of the JmjC domain has significant similarity with members of the cupin superfamily that are frequently metalloenzymes with zinc-ion-containing active sites (Clissold and Ponting, 2001). The typical human proteins that contain the JmjC

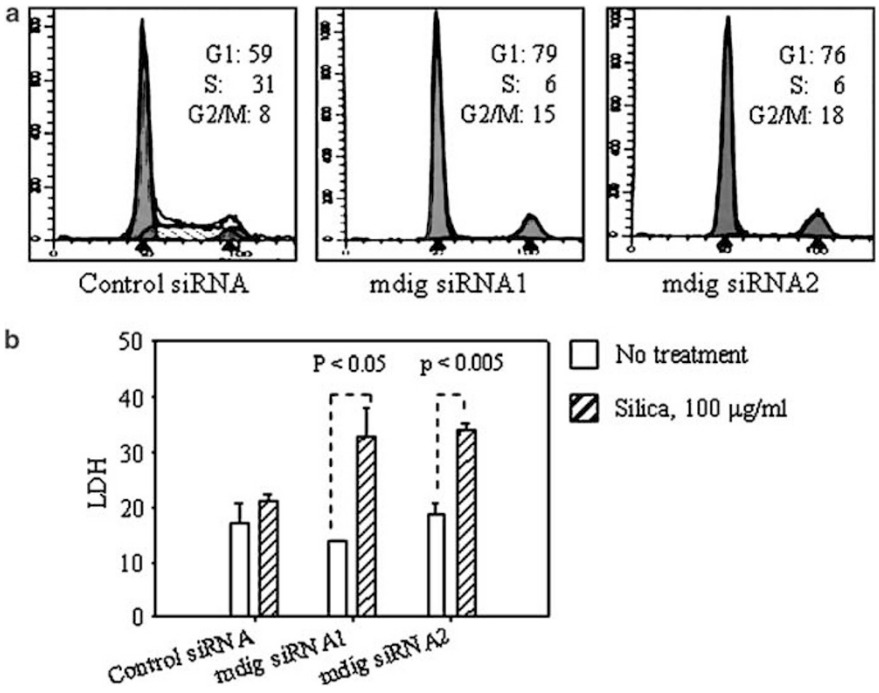

Figure 8 Silencing of *mdig* mRNA delays cell cycle transition and enhances silica-induced cytotoxicity. (**a**) Flow cytometry study of the cells transfected with the control siRNA or *mdig* siRNA. (**b**) Cytotoxicity assay of the cells transfected with the indicated siRNA for 24 h and then cultured in 0.1% of serum in the absence or presence of silica for an additional 18 h. Data show relative units of LDH measured in the cell culture medium ($n = 3$)

domain include human *hairless*, phosphatidylserine receptor and retinoblastoma-binding protein2, all of which have been associated with the protection or regulation of cell apoptosis or survival (Panteleyev *et al*., 2000; Savill *et al*., 2003). Consistent with the function of these human JmjC-containing proteins, when the expression of *mdig* mRNA is repressed by siRNA, cells undergo decreased proliferation and an enhanced silica-induced cytotoxicity, cellular events that support a functional role of the *mdig* gene in cell growth and survival.

When we first cloned the *mdig* gene and deposited the partial sequence of *mdig* into the GenBank (GenBank Access number: BE441202, released on July 25, 2000), there was no reported DNA sequence homology in the known gene database of GenBank, suggesting that the *mdig* gene was indeed a novel gene. Upon further characterization of the *mdig* gene, Tsuneoka *et al*. (2002) recently reported an Myc-inducible gene, *mina53* (GenBank Access number: AB083189, released on September 25, 2002) that is expressed in human glioblastoma cell line T98G that was engineered to ectopically express c-Myc. The DNA sequence of *mina53* is 99% identical to that of *mdig*, suggesting that *mina53* and *mdig* are from the same gene loci. C-Myc regulation of *mina53* is believed to occur through the two c-Myc-binding elements in the 5′-untranslated region of the *mina53* gene. In our own hands, however, we found that the c-Myc-binding elements are dispensable for both basal and silica-induced *mdig* expression. Instead, the c-Ets-1 and three Sp-1 sites in the −300 to −500 region from the transcription start site are crucial for the basal expression of *mdig*, whereas the AP-1-binding site at −795 and NF-κB-binding site at −198 may contribute to the inducible expression of *mdig* by silica particles and H_2O_2 (Zhang *et al*, unpublished). Our finding that most lung cancer tissues or lung cancer cell lines overexpress *mdig* (Figure 5) and the observation suggesting a proliferative role of *mdig* in A549 cells (Figure 7) indicates that *mdig* may be associated with carcinogenesis.

An additional intriguing finding from the present study is the detection of the alternative spliced transcripts of *mdig* mRNA in some lung cancer cell lines. In the non-small-cell lung cancer cell line (H441), the *mdig* mRNA, named *mdig-2,* lacks the entire exon 2 region and has a 207 bp insert between the exon 5 and exon 6 region. The insert between the exon 5 and exon 6 region has also been observed in a transcriptional variant of *mina53* (GenBank Access number: AB083191). However, the insert in the *mina53* variant does not contain the first 99 bp observed in the insert of *mdig-2*. In addition, the *mina53* variant is different from *mdig-2* by the inclusion of partial exon 2 region. The presence of alternative spliced mRNA has been linked to malignancy due to aberrant splicing and abnormal protein production (Garcia-Blanco *et al*., 2004). The most common form of alternative splicing is the inclusion or the skipping of one or more exons. Alternative splicing in the nontranslational region may change the rate of transcription/translation and the stability of mRNA, whereas the alternative splicing in the coding region will result in the generation of

different protein isoforms or initiation of nonsense-mediated mRNA decay (Maquat, 2004). Detailed functional analysis of alternative splicing of *mdig* mRNA will be pursued in future studies.

Materials and methods

Cells and reagents

Human AMs were collected from the recruited volunteers according to the protocols approved by the Institutional Review Board. To avoid complexity of cigarette smoking-related diseases, all of the volunteers were nonsmokers with a normal lung function (Table 2). AMs were separated from bronchial lavage fluid by centrifugation as described by Weissman *et al*. (1986). The cell pellets were washed twice with PBS and resuspended in DMEM containing 10% heat-inactivated FCS, 100 IU/ml penicillin G, and 50 μg/ml gentamicin. AMs were purified by adherence for 90 min at 37°C under a 95% air and 5% CO_2 atmosphere in DMEM medium in 75-cm^2 culture flasks. Human lung cancer tissues and their corresponding surrounding noncancerous tissues were obtained from patients who underwent surgical removal of lung cancer. The A549 and other lung cancer cell line were purchased from the American Tissue Culture Collection (Manassas, VA, USA). Reagents for the DDRT–PCR, pCR-TRAP cloning system, and MessageClean Kit were obtained from GenHunter Corporation (Nashville, TN, USA). The SequaGel sequencing system for the preparation of the nucleic-acid sequencing gel was purchased from National Diagnostics (Atlanta, GA, USA). Radioisotope ^{33}P-nucleotide was purchased from DuPont NEN (Boston, MA, USA). The DNA sequencing kit was obtained from Amersham Life Science (Piscataway, NJ, USA). The RT–PCR kit was purchased from Promega (Madison, WI, USA).

DDRT–PCR

Total RNA was extracted from human AMs or tissue samples by the method described by Chomczynski and Sacchi (1987) and Liang *et al*. (1992). DNA-free total RNA (2 μg/sample) was subjected to DDRT–PCR according to protocols described by Liang *et al*. (1992). The DDRT–PCR products from different primer combinations were separated in nondenaturing 4.5% polyacrylamide sequencing gels and visualized by autoradiography. Bands exhibiting differential expression between RNA samples from normal AMs and miner AMs were excised from the dried gels and reamplified using the appropriate primers. The reamplification of DDRT–PCR bands of interest was confirmed by electrophoresis in an agarose gel. The PCR product was subcloned into a pCR-TRAP cloning vector and sequenced. The DNA sequence data were compared through a BLAST search in GenBank.

The PCR primers used for the amplification of *mdig* mRNA fragments are: *mdig*-a: 5′-TCA TGT CGG GCC TAA GAG AC-3′; *mdig*-b: 5′-GGC ATT TGA TTC TGC AAA GG-3′; *mdig*-c: 5′-ATG GGT CCC TGT TCA AGC TA-3′; *mdig*-d: 5′-GGA GTG TCC GCT TGA TGA AT-3′. For the determination of alternative splicing of *mdig* mRNA, different combination of above PCR primers was used as follows: PCR primer set I: *mdig*-a + *mdig*-b; PCR primer set II: *mdig*-c + *mdig*-d; PCR primer set III: *mdig*-a + *mdig*-d; PCR primer set IV: *mdig*-c + *mdig*-b.

Northern blot

The human multiple tissue Northern blot membrane (MTN), SpotLight random primer labeling kit, SpotHyb buffer, and SpotLight detection kit were purchased from Clontech (Palo Alto, CA, USA). The MTN blot contains ~2 μg of poly(A)$^+$ RNA per lane from eight different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas). The cDNA probes for human *mdig* and β-actin were labeled with biotin using the SpotLight chemiluminescent random primer labeling kit. The membrane was prehybridized for 30 min at 45°C in 10 ml of SpotHyb buffer and hybridized with the biotinylated cDNA probes overnight under the same conditions. At the end of hybridization, the membranes were incubated with stabilized streptavidin–HRP conjugate in blocking buffer for 15 min at room temperature. The hybridization signals were visualized by incubation of the membranes with a chemiluminescent detection system for 5 min at room temperature and exposure to X-ray film for 1 min.

Gene knockdown by small interfering RNA (siRNA)

The selection of siRNA targeting sequences against human *mdig* was performed according to the criteria described by Reynolds *et al*. (2004) using an siRNA design program, Gene-specific siRNA selector, developed by Wistar Bioinformatics (http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAindex.htm). To ensure efficacy of gene knockdown by siRNA, the target accessibility of *mdig* mRNA was analysed by a Sfold

Table 2 Data summary of human subjects

	Control	*Active miners*	*Inactive miners*
Numbers	35 males	11 males	11 males
Age (years)	28 (4.1)	27 (8.1)	24 (3.7)
Dyspnea	1/35	6/11	11/11
Smoking	No	No	No
Cough	1/35	4/11	9/11
Sputum	0/35	6/11	7/11
FVC (% predicted)	103.3 (2.2)	99.1 (4.1)	101.8 (3.3)
FEV_1/FVC	78.1 (1.2)	76.3 (1.4)	76.0 (1.6)
TLC (% predicted)	103.7 (2.2)	100.9 (3.2)	102.2 (2.3)
D_LCO(% predicted)	127.3 (2.8)	111.6 (7.1)	118.5 (5.4)
D_CCO/VA (%)	115.1 (2.4)	114.7 (3.5)	117.1 (3.8)
BAL cell recovery	15.9 (1.7) $\times 10^6$	35.5 (6.1) $\times 10^6$	19.7 (2.7) $\times 10^6$

Note: Data presented as mean $\pm$ (s.e.m.); FVC, force vital capacity; FEV_1/FVC, force expiratory volume in 1 s/forced vital capacity; TLC, total lung capacity; D_LCO, single breath carbone monoxide diffusing capacity; VA, alveolar volume

program that predicts potential secondary structures of mRNA, including hairpin, bulge, and multibranched structures (http://sfold.wadsworth.org/sirna.pl). Two different siRNAs designated *mdig siRNA1* and *mdig siRNA2*, which target nucleotides 1084–1104 and 1221–1241 of the human *mdig* mRNA sequence (GenBank Access number: AY302110), respectively, were synthesized and HPLC purified by Qiagen (Valencia, CA, USA). The 21 nt siRNA against luciferase (provided by Qiagen), a nonmammalian protein from *Photinus pyralis* (American firefly), was used as a control to determine the potential presence of nonspecific effects of siRNA. The targeting sequences of siRNA are: *mdig siRNA1*: AAGAACTGCTTTCCTCAGACA; *mdig siRNA2*: AAAGACCACATTGTCCTCACA. A549 cells were seeded in six-well plates on the day before transfection at a concentration of 5×10^5/ml cells. Cells were transfected with siRNA using RNAiFect reagent (Qiagen) according to the manufacturer's instructions at a final siRNA-duplex concentration of 400 nM.

Cytotoxicity assay

Cytotoxicity was determined by the release of cellular LDH, which served as an indicator of cytotoxicity in the cells transfected with different siRNA in the absence or presence of additional stimulators. The cells were cultured in 24-well plates, and the media were collected after 24–48 h of siRNA transfection. LDH activity released from the cytosol of damaged cells was quantitated using a Roche LDH reagent (Roche, Indianapolis, IN, USA) on a Roche COBAS MIRA chemistry instrument according to the manufacturer's instructions.

Image analysis

The cells transfected with siRNAs were cultured in six-well plates. The formation of cell confluence was monitored after 24 h of siRNA transfection using a Zeiss Axiovert100 microscope at a magnification of $\times 20$. The image was captured with a Pixera Pro150ES digital camera connected to the microscope and operated by Pixera Viewfinder3.0 software. Four random areas in each well were selected. Each picture was converted in gray scale and saved in TIF format and analysed by an evaluation model of TotalLab v2003.3 colony counting software provided by Nonlinear Dynamics Ltd. (Newcastle, UK). The data were expressed as cell numbers per artificial field as determined by the software.

Statistical analysis

The measurement errors for the quantitative experiments were determined using coefficient of variation (Cv = standard deviation (δ)/mean (μ)). The sample size for cell proliferation and LDH activity was based on obtaining a power of at least 0.9 at a 0.05 significance level. The data were expressed as mean $\pm$ s.d. SigmaStat software was used for general statistical analysis.

Acknowledgements
This work was partially supported by the United States Bureau of Mines, Generic Mineral Technology Center for Respiratory Dust Grant G1145242 to LM Demers and a Career Development Award under a cooperative agreement from the Centers for Disease Control and Prevention through the Association of Teachers of Preventive Medicine to F Chen. The DDRT–PCR, cloning, and DNA sequencing were conducted by Y Lu and F Chen when both of them were employees of the Pennsylvania State University College of Medicine at Hershey during July, 1994 to June, 1998. We thank Dr Murali Rao and Mr Terry Meighan in the Pathology and Physiology Research Branch at the National Institute for Occupational Safety and Health for their gift of total RNA from A549 cells and the *mdig* siRNA they purchased.

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